AD_____

Award Number: DAMD17-99-1-9110

TITLE: Src-JNK Potentiation of Estrogen Receptor AF-1; Mechanism, and Role in Estrogen Action in Breast Cancer

PRINCIPAL INVESTIGATOR: Peter J. Kushner, Ph.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0549

REPORT DATE: August 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2001	Annual (1 Aug 00 - 31 Jul 01)

4. TITLE AND SUBTITLE
Src-JNK Potentiation of Estrogen Receptor AF-1; Mechanism, and Role in Estrogen Action in Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9110

6. AUTHOR(S)
Peter J. Kushner, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, San Francisco
San Francisco, California 94143-0549

E-Mail: kushner@itsa.ucsf.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The activity of Src tyrosine kinase is commonly elevated in breast cancer and breast cancer cell lines, but the significance of this elevation is not known. In preliminary studies we found that increasing Src activity potentiates the ability of the estrogen receptor to stimulate transcription of target genes, and thereby alter cellular functions, and that Src must activate the MAP kinase proteins JNKs and ERKS in order to potentiate the estrogen receptor. We have two objectives. One is to understand in detail the molecular pathway whereby activated Src and JNK (and also ERKS) leads to an increase in estrogen receptor activity. A second objective is to understand the potential role of Src in estrogen induced mammary ductal development and estrogen-induced breast cancer proliferation.

In the second year of this study we have accomplished and added unexpected further progress to the first of these objectives. We found that Src activated the first 100 amino acids in the estrogen receptor AF-1 function as its main target, that to do so Src worked primarily through the JNK family of MAP kinases, and that the JNK target appears to be the p160 coactivators coactivators that mediate AF-1 action. We have also found that ERKs target a specific residue S763 in the p160 coactivator. Are findings have just been published in two articles. These results suggest potential new targets for anticancer therapy.

14. SUBJECT TERMS
Breast Cancer/Src tyrosine kinase/estrogen receptor/JNK/AF-1

15. NUMBER OF PAGES
29

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

.....

Introduction, Src year 1 annual report

The activity of Src is commonly elevated in breast cancer and breast cancer cell lines, but the significance of this elevation is not known. Many breast cancers and breast cancer cell lines also contain estrogen receptors and are stimulated to grow by estrogen. In preliminary studies we found that increasing Src activity potentiates the ability of the estrogen receptor to stimulate transcription of target genes. These prelimary studies suggest a possible connection between Src activity and estrogen receptor activity that we propose to explore. We have two objectives. One is to understand in detail the molecular pathway whereby activated Src and JNK leads to an increase in estrogen receptor activity. A second objective is to understand the potential role of Src in estrogen induced mammary ductal development and estrogen-induced breast cancer proliferation.

(5) BODY
We had three tasks:

1. To identify the pathway leading from Src activated JNK to increased estrogen receptor AF-1 activity (months 1-18).
2. To determine the role of Src in estrogen induced mammary gland ductal epithelial proliferation and branching at puberty. (months 24-36)
3. Determine the role of Src in estrogen stimulated tumor growth by studying the effects of estrogen on the proliferation of breast cancer cell lines in which Src has been made nonfunctional (months 24-36)

In this, the second year of the project, again most of our progress has been on task 1. Our progress is detailed in two appended manuscript published studies.

Potentiation of estrogen receptor AF-1 by Src/JNK through a serine 118-independent pathway.

The above study appeared in Molecular Endocrinology in January of 2001.[1] In this study we showed that activated Src increases the activity of the estrogen receptor through activation of Jun N-terminal Kinases (JNKs). We also show that Src activates estrogen receptor through the extracellular receptor activated kinases (ERKs), which are also activated by a variety of growth factors such as epidermal growth factor (EGF). Neither of these pathways was blocked by mutations in the estrogen receptor that prevented phosphorylation, and indeed the direct target of the phosphorylation cascades appears to be the p160 coactivators such as GRIP1. We have detailed the pathway from ERKs to GRIP1 and then to steroid receptors in the second published study.

Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity.

The above study was published in the Journal of Biological Chemistry, June 2001[2]. We have since found evidence that the JNK pathway also targets the p160 coactivators and are preparing a manucsript.

We have just begun to work on task 2. On Task 3, as described in the published paper by Feng et al.[1], we found that in MCF-7 breast cancer cells that the activity of the endogenous ER was enhanced by elevating Src activity by transfection with v-Src. This establishes that in breast cancer cell lines that ER activity is under Src regulation, and provides a critical control for the experiment in which we determine whether reducing Src activity in these cells reduces ER action on transcription and proliferation.

(6) KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that the Src tyrosine kinase, known to be elevated in breast cancer, increase the activity of the estrogen receptor, a critical proliferative factor in many breast cancers.

- Demonstrated that the pathway from Src leads to JNK, a MAP kinase,ERK another MAP kinase and then to estrogen receptor activator function 1, almost certainly through the p160 coactivators especially GRIP1.

- Demonstrated that increased Src activity potentiates estrogen action in human breast cancer cells in culture.

- Published these findings in two peer reviewed publications.

(7) REPORTABLE OUTCOMES.

1. Feng W; Webb P; Nguyen P; Liu X; Li J; Karin M; Kushner PJ. Potentiation of estrogen receptor activation function 1 (AF-1) by Src/JNK through a serine 118-independent pathway. Molecular Endocrinology, 2001 Jan, 15(1):32-45.

2. Lopez GN; Turck CW; Schaufele F; Stallcup MR; Kushner PJ. Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. Journal of Biological Chemistry, 2001 Jun 22, 276(25):22177-82.

Copies of these are appended.

(8) CONCLUSIONS;

Our main conclusions are first, that the Src tyrosine kinase, known to be elevated in breast cancer, increases the activity of the estrogen receptor, a critical proliferative factor in many breast cancers. This activity of Src is seen in transfected cells of various kinds and also seen in MCF-7 cells expressing their own estrogen receptor. This conclusion suggests that overactivity of Src in breast cancer may be leading to hyper activity of the estrogen receptor and may be playing a key role in estrogen dependent breast cancer. "So what?" If the conclusion is correct it will suggest new ways to intervene in breast cancer prevention and therapy.

We also conclude that the pathway from Src leads to JNK and ERKs, both MAP kinases. It then proceeds to p160 coactivators, to estrogen receptor, and to genes under estrogen receptor control. "So what?" These conclusions suggest that drugs that might target any of the steps in this pathway, might be useful as potential treatments for hormone dependent breast cancer.

(9) REFERENCES

1. Feng W; Webb P; Nguyen P; Liu X; Li J; Karin M; Kushner PJ. Potentiation of estrogen receptor activation function 1 (AF-1) by Src/JNK through a serine 118-independent pathway. Molecular Endocrinology, 2001 Jan, 15(1):32-45.

2. Lopez GN; Turck CW; Schaufele F; Stallcup MR; Kushner PJ. Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. Journal of Biological Chemistry, 2001 Jun 22, 276(25):22177-82.

Potentiation of Estrogen Receptor Activation Function 1 (AF-1) by Src/JNK through a Serine 118-Independent Pathway

Weijun Feng, Paul Webb, Phuong Nguyen, Xiaohong Liu, Jiandong Li, Michael Karin, and Peter J. Kushner

Metabolic Research Unit (W.F., P.W., P.N., P.J.K.) and
Department of Anatomy (J.L.)
University of California
San Francisco, California 94143

Department of Pharmacology (X.H.L., M.K.)
University of California San Diego
La Jolla, California 92093

Estrogen receptor (ER) is activated either by ligand or by signals from tyrosine kinase-linked cell surface receptors. We investigated whether the nonreceptor Src tyrosine kinase could affect ER activity. Expression of constitutively active Src or stimulation of the endogenous Src/JNK pathway enhances transcriptional activation by the estrogen-ER complex and strongly stimulates the otherwise weak activation by the unliganded ER and the tamoxifen-ER complex. Src affects ER activation function 1 (AF-1), and not ER AF-2, and does so through its tyrosine kinase activity. This effect of Src is mediated partly through a Raf/mitogen-activated ERK kinase/extracellular signal-regulated kinase (Raf/MEK/ERK) signaling cascade and partly through a MEKK/JNKK/JNK cascade. Although, as previously shown, Src action through activated ERK stimulates AF-1 by phosphorylation at S118, Src action through activated JNK neither leads to phosphorylation of S118 nor requires S118 for its action. We therefore suggest that the Src/JNK pathway enhances AF-1 activity by modification of ER AF-1-associated proteins. Src potentiates activation functions in CREB-binding protein (CBP) and glucocorticoid receptor interacting protein 1 (GRIP1), and we discuss the possibility that the Src/JNK pathway enhances the activity of these coactivators, which are known to mediate AF-1 action. (Molecular Endocrinology 15: 32–45, 2001)

INTRODUCTION

The action of estrogen receptor-α (hereafter, ER) is regulated both by binding of ligand and by inputs from signal transduction cascades. Binding of estrogen to ER frees it from a complex with heat shock proteins and allows ER to bind estrogen response elements (EREs) in the promoter region of target genes (for review see Ref. 1). ER then stimulates transcription via the concerted action of the AF-1 activation function in its amino terminus and the hormone-dependent activation function, AF-2, that lies within the ligand-binding domain (LBD). The antiestrogen tamoxifen allows release from heat shock proteins and ERE binding, but blocks AF-2 (reviewed in Refs. 1 and 2). Tamoxifen allows weak AF-1 activity, but in many cases this is insufficient to increase gene expression (3). Other antiestrogens, such as raloxifene and ICI 182,780 (ICI), allow neither AF-1 nor AF-2 activity (4, 5). Both activation functions work by recruiting a coactivator complex to the promoter (reviewed in Refs. 1, 6, and 7). The complex contains a p160 protein, such as SRC-1(N-CoA1) (8, 9), glucocorticoid receptor interacting protein 1 (GRIP1) (TIF2, N-CoA2) (10–12), or p/CIP (AIB1, ACTR, TRAM-1, RAC3) (13–17), p300/CBP (CREB binding protein) (9, 18–20), and p/CAF (21–23) (for review see Ref. 7). AF-1 binds the C terminus of the p160 component (24), whereas estrogen-bound AF-2 binds tightly to a short α-helices with the consensus LXXLL (the NR boxes) that are repeated several times within each p160 (13, 25–29). The coactivator complex, once recruited, stimulates transcription via its histone acetyl-transferase activity, which is thought to remodel chromatin and allow access to the transcriptional template, and by interactions with the basal transcription machinery (15, 30–32).

ER activity is also stimulated by signaling pathways that are activated when growth factors, such as epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I), bind their tyrosine kinase-linked receptors. Growth factors are sometimes sufficient to acti-

0888-8809/01/$3.00/0
Molecular Endocrinology 15(1): 32–45

vate ER in the apparent absence of ligand (33–36). More commonly, growth factors synergize with ligand by enhancing AF-1 activity. EGF binding to the EGF receptor results in sequential activation of Ras, Raf, MEK, and the mitogen-activated protein (MAP) kinases ERK1 and ERK2, which phosphorylate ER at serine 118 in the N-terminal domain and potentiate AF-1 activity (36–38). Mutation of S118 to alanine blocks ER phosphorylation by MAP kinases and potentiation of AF-1 action by growth factors (36–38). Furthermore, the S118A mutation also reduces basal phosphorylation of S118 by unspecified kinases (38) and decreases basal AF-1 activity (39, 40). Phosphorylated ER AF-1 shows enhanced binding to the p68 RNA helicase, which is thought to account for its enhanced transcriptional activity (41). AF-1/GRIP1 interactions map to the N-terminal region of the ER AB domain, which has not been implicated in growth factor enhancement of AF-1 activity, and are unaffected by mutations in serine 118 (24).

ER is likely to be subject to signal transduction inputs during normal development and repair processes and may also be subject to abnormal stimulation by such pathways in pathological states, such as cancer. Many breast tumors exhibit elevated expression of growth factors such as EGF, Her2/neu, IGFs, and their receptors (42–45). Furthermore, more than 80% of primary breast cancers show increased activity of the nonreceptor Src tyrosine kinase activity compared with normal breast tissue (Refs. 46–49; reviewed in Ref. 50). Elevated Src activity leads to activation of multiple signal transduction cascades (reviewed in Refs. 51–53) that, in turn, activate both the ERK and JNK subgroups of MAP kinases (for examples see Refs. 54 and 55). Src activates ERKs presumably via Ras and the Raf/MEK/ERK kinase cascade (see, for example, Ref. 54). It also activates JNKs, presumably via Rac-1 and related GTPases, and the sequential activation of the MEKK/JNKK/JNK kinase cascade (56). In light of these reports we investigated whether activated Src enhances ER action. We find that Src does so and that it specifically enhances ER AF-1 activity via two independent mechanisms. One involves phosphorylation at S118 via Src activation of the Raf1-MEK-ERK pathway. The other is mediated by Src activation of the MEKK-JNKK-JNK pathway, the target of which does not appear to be S118, or even ER. We discuss the possibility that JNKs target one of the several coactivators that associate with the ER.

RESULTS

Src Potentiates ER AF-1

Src activity in breast cancer cells and cell lines is often elevated up to 30-fold (49). To examine the effects of elevated Src activity on ER action, we transfected expression vectors for v-Src and control vectors into HeLa cells along with expression vectors for ER and an ERE responsive reporter gene (ERE:HSV-TK-CAT). v-Src is a viral derivative of cellular Src (c-Src) which has at least 10-fold higher kinase activity than c-Src. Figure 1A shows that v-Src potentiated ER transcriptional activity by 2-fold in the presence of 17β-estradiol (E_2), a primary estrogen agonist activating both AF-1 and AF-2. V-Src also potentiated ER activity in the absence of ligand by about 2-fold. More strikingly, v-Src enhanced ER transcriptional activity in the presence of tamoxifen (Tam), an ER ligand that inhibits AF-2 but allows AF-1 activity (3), by about 15-fold. V-Src had no effect upon ER transcriptional activity in the presence of ICI 182,780, an ER ligand that blocks the activities of both AF-1 and AF-2 (3, 57–59). Thus, v-Src increases ER transcriptional activity in the presence of estradiol and the absence of ligand, but shows larger effects in the presence of tamoxifen. We then examined v-Src effects upon ERG400V, an ER mutant that lacks constitutive activity but is otherwise normal (60). Here, v-Src showed modest potentiation of estrogen response and a much larger potentiation of tamoxifen response. Thus, v-Src truly enhances the transcriptional activity of the tamoxifen-ER complex above basal. Over the course of this study, v-Src enhanced the overall levels of estrogen response by between 50% and 4-fold, but consistently yielded larger enhancements of tamoxifen response. Thus, v-Src increased the overall level of tamoxifen response from a tiny percentage of estrogen response to 15–40% of estrogen response.

To confirm that v-Src effects upon ER activity could not be simply explained by increases in ER levels, we performed Western blots on extracts of cells that had been transfected with different amounts of ER in the presence and absence of transfected v-Src. Figure 1B shows that the amount of ER increased as a function of transfected ER expression vector in the absence of v-Src, and that v-Src increased ER levels by about 3-fold. In parallel, v-Src gave much stronger potentiation of ER action at the ERE responsive reporter gene, even at ER levels that were optimal for estrogen and tamoxifen response (Fig. 1B, *lower panel*). In particular, the ER activity obtained with 3 μg of transfected ER and v-Src exceeded the ER activity obtained with 10 μg of transfected ER and no v-Src, even though the former contained lower amounts of ER protein. Thus, v-Src increases ER transcriptional activity, especially in the presence of tamoxifen.

We then asked whether v-Src enhancement of ER activity might occur under more physiological conditions. We first examined the amount of v-Src required for enhancement of ER activity. Tamoxifen response increased with as little as 300–600 ng of transfected v-Src expression vector (Fig. 1C). We then examined v-Src action upon the ERE responsive reporter in MCF-7 breast tumor cells, which express endogenous ER (Fig. 1D). Here, ER showed significant constitutive activity, which was further elevated by addition of estradiol but completely suppressed by tamoxifen and

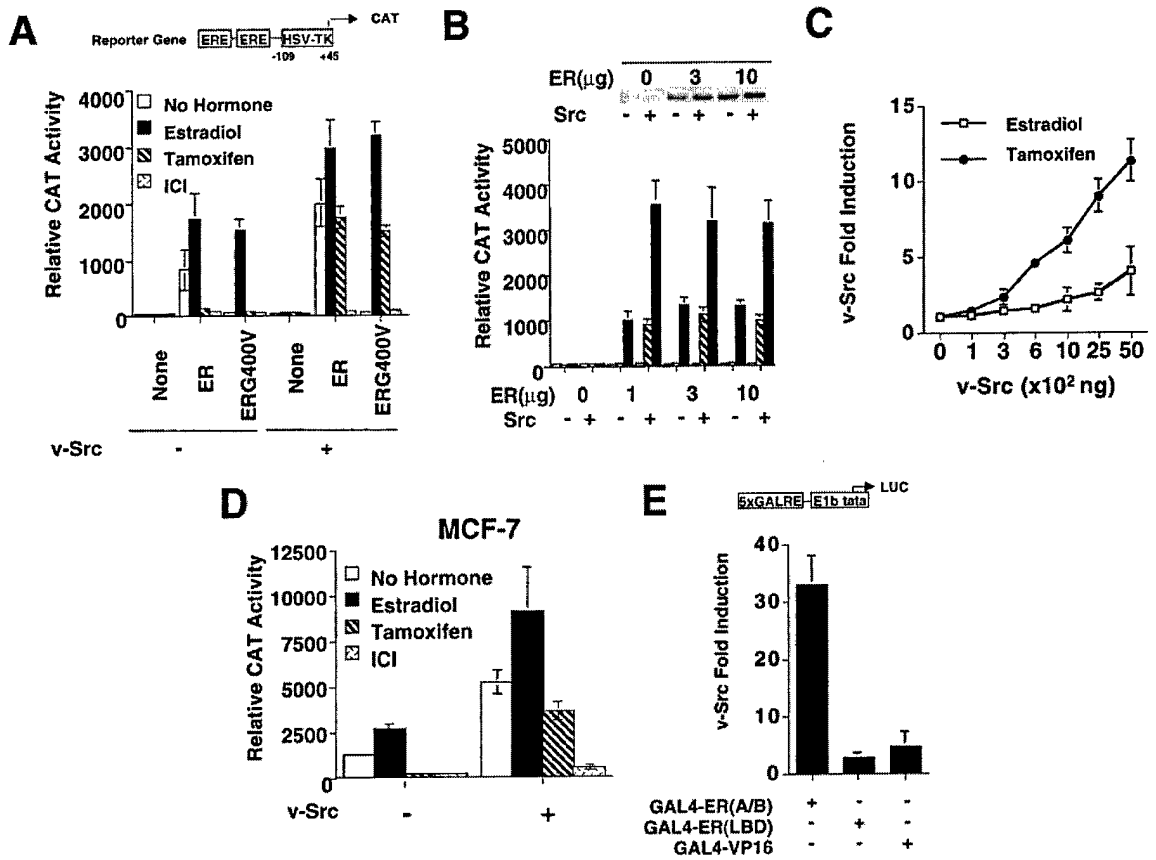

Fig. 1. v-Src Potentiates ER AF-1 Transcriptional Activity

A, Activity of an ERE-CAT reporter gene in HeLa cells transfected with expression vectors for wild-type ER, the ER G400V derivative, and v-Src and treated with estradiol (E_2), tamoxifen (Tam), and ICI 182,780 (ICI). B, Western blots were performed upon extracts of cells that were transfected with different amounts of ER expression vector, or control vector, ±transfected v-Src. The *lower panel* shows transcriptional activation of the ERE responsive reporter gene as a function of ER levels ± transfected v-Src. C, Activity of the ERE reporter gene in HeLa cells transfected with an expression vector for ER, the indicated amount of expression vector for v-Src after treatment with tamoxifen (*closed circles*) or vehicle (*open boxes*). Fold inductions were determined by comparing the relative CAT activity in the presence of v-Src to that in the absence of v-Src (set as 1-fold). D, Activity of the ERE-CAT reporter gene in MCF-7 breast tumor cells that express endogenous ERs. E, Activity of a reporter gene with five GAL4 response elements in cells transfected with expression vectors for GAL4 fusion proteins to the ER A/B region (GAL4-AB), to the ER ligand binding domain (GAL-LBD), or to the herpes virus VP16 activation domain (GAL4-VP16) in the presence or absence of cotransfected v-Src. Fold inductions were determined as in Fig. 1C.

ICI. In the presence of transfected v-Src, both the constitutive and estrogen-dependent transcriptional activity were modestly elevated and, once again, tamoxifen-dependent transcriptional enhancement was strongly increased. Thus, v-Src enhances the activity of the estrogen-ER and tamoxifen-ER complexes in breast cells, just as it does in HeLa cells.

That the ER response to v-Src overexpression is more dramatic in the presence of tamoxifen than in the presence of estrogen, and that there is no response in the presence of ICI, suggests that ER AF-1 might be a primary action target of v-Src. To investigate whether the target of v-Src action was AF-1 or AF-2, we examined the effect of v-Src overexpression upon the activity of a reporter gene with a promoter containing a TATA box and multiple binding sites for the yeast GAL4 protein (5xGALRE-E1b-tata-LUC). This reporter was then activated with the DNA binding domain of GAL4 fused to the ER A/B region containing AF-1 [GAL4-ER(A/B)], to the ER LBD containing AF-2 [GAL4-ER(LBD)], or to VP16 (GAL4-VP16). As shown in Fig. 1E, v-Src potentiated the GAL4-ER(A/B) transcriptional function approximately 30- to 40-fold, but had only a minimal effect on GAL4-LBD or GAL4-VP16. Like v-Src action upon the tamoxifen-liganded ER, v-Src potentiation of AF-1 activity could be detected at optimal levels of transfected GAL4-ER(A/B) expression vector (data not shown). Thus, v-Src strongly enhances ER AF-1 activity. These observations are consistent with the strong activation of the tamoxifen-bound ER by v-Src and confirm that the A/B domain of ER, which contains AF-1, is the v-Src target.

Src Action upon ER Is Independent of Tyrosine 537

Some reports have suggested that Src directly phosphorylates ER at tyrosine 537 (Y537) within the ER-LBD (61–63), although the physiological significance of this effect is unclear. In fact, some Y537 mutations were later shown to enhance ER activity in the absence of hormone, by allowing ligand-independent interactions of ER AF-2 with its target coactivators (64–66). To determine whether Y537 phosphorylation was required for v-Src action upon the ER, we examined the effect of v-Src overexpression upon several ERs bearing mutations at Y537. We chose Y537R, which shows similar constitutive activity to wild-type ER, and Y537S and Y537F, which show either enhanced or reduced constitutive activity, respectively.

Figure 2A shows that ER and ER-G400V elicited strong estrogen responses and weak tamoxifen responses from the ERE responsive reporter. In parallel, ERY537R showed comparable constitutive activity to wild-type ER, and ERY537S showed the expected increase in constitutive activity, but both mutants showed similar levels of activity to wild-type ER in the presence of estrogen and tamoxifen. In the presence of v-Src, wild-type ER and each Y537 mutant (Y537R and Y537S) showed a modest enhancement of transcriptional activity in the presence and absence of estradiol and a larger enhancement of transcriptional activity in the presence of tamoxifen. Thus, v-Src effects upon the estrogen-ER complex, the unliganded ER, and the tamoxifen-ER complex are all independent of a requirement for ER phosphorylation at tyrosine 537. v-Src also enhanced estradiol and tamoxifen activation in the presence of the ERY537F mutant, which lacks constitutive activity (Fig. 2B). This confirms that v-Src enhances the activity of the tamoxifen-ER complex over basal, even when tyrosine 537 is mutated. We conclude that v-Src enhancement of ER activity occurs through effects upon AF-1, and not through effects upon tyrosine 537 in the ER-LBD.

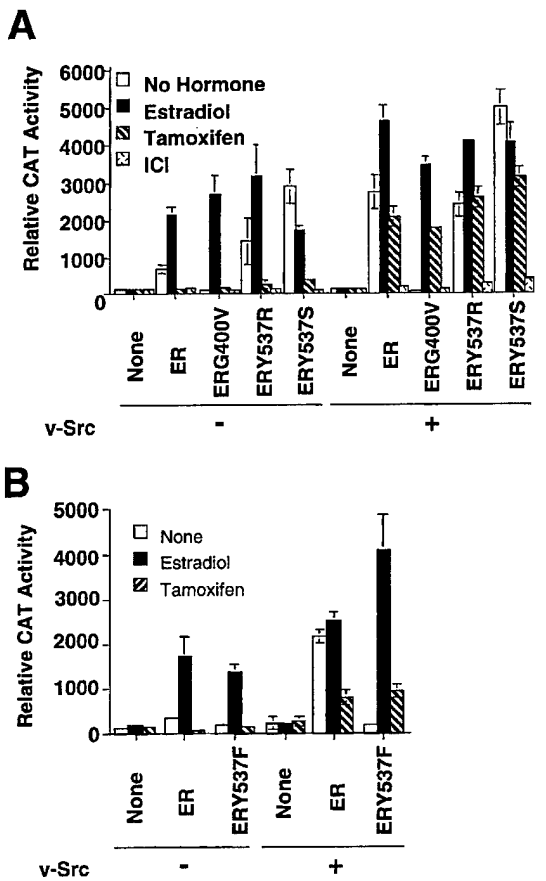

Fig. 2. Src Action upon ER Is Independent of Tyrosine 537

A, Activity of the ERE-CAT reporter was determined in the presence of empty pSG5 expression vector, or expression vectors for ER, ERG400V, ERY537R, and ERY537S, both in the presence of an empty expression vector or an expression vector for v-Src. B, A similar experiment performed with empty CMV expression vector or expression vectors for wild-type ER and the Y537F derivative.

Src Kinase Activity Is Required for Potentiation of ER AF-1

To investigate whether elevated Src kinase activity itself, and not some other feature of v-Src, leads to potentiation of ER action, we examined the effect of various Src expression vectors and tyrosine kinase inhibitors upon ER AF-1 activity. Wild type c-Src and constitutively activated c-Src (Y527F) potentiated the activity of the GAL-ER(A/B) fusion protein by 2~3- and 5-fold, respectively (Fig. 3). The kinase-inactive mutant Src (Y295K, Y527F), in contrast, was unable to enhance AF-1. Moreover, enhancement of ER AF-1 by Src was blocked, and even reduced below basal levels, by genistein and herbymicin A, two inhibitors of Src tyrosine kinase activity (Fig. 3). Control experiments indicated that both of these inhibitors were without effect on GAL-VP16 (data not shown). These results indicate that the intrinsic tyrosine kinase, and not another feature of the v-Src molecule, mediates the enhancement of ER activities. That the kinase inhibitors reduce AF-1 level below basal may further suggest that endogenous Src tyrosine kinase activity might underlie basal AF-1 activity.

Src Potentiates AF-1 through Both the Ras-Raf1-MEK-ERK and the Rac-MEKK-JNKK-JNK Pathways

Src tyrosine kinase might affect AF-1 either directly or indirectly through the numerous signal transduction cascades that Src is known to initiate. While Src is generally associated with the plasma membrane, and ER is generally nuclear, these locations are not rigid. In particular, there are reports of ER associated with the cell surface (67, 68). Thus, there is nothing *a priori* to exclude the possibility that Src might directly phos-

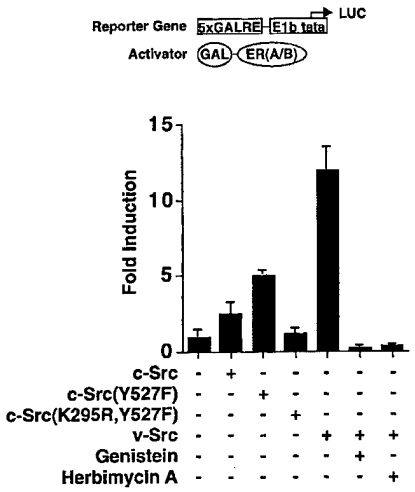

Fig. 3. Src Action on ER AF-1 Requires Src Tyrosine Kinase Function

Shown is fold induction of the GALRE reporter gene activated by GAL-ER(A/B) in the absence of Src (normalized to 1) and by c-Src, by constitutively active c-Src [c-Src (Y527F)], by an inactive derivative of c-Src [c-Src(K295R, Y527F)], or by v-Src in the absence or presence of tyrosine kinase inhibitors (genistein or herbimycin). The effect of the inhibitors on v-Src-enhanced and basal stimulation by GAL-Elk1is shown in the *inset*.

phorylate the ER AF-1 domain. We therefore tested whether a glutathione-*S*-transferase (GST)-ER(A/B) fusion protein was a substrate for activated c-Src kinase. GST-ER(A/B) was efficiently phosphorylated by activated ERK2 MAP kinase, consistent with other studies (37), but not by activated c-Src kinase (Fig. 4A). Thus, ER is not a direct substrate of Src. We infer that Src must activate ER AF-1 indirectly through phosphorylation cascades.

Because Src is known to cause MAP kinase activation, and the well known pathway of Ras, Raf-1, MEK, and ERK activation enhances AF-1 activity through serine 118 phosphorylation, we asked whether v-Src might enhance ER activity by initiating the Raf-1/MEK/ERK cascade. We first examined the effect of various inhibitors of this pathway upon EGF stimulation of the transcription factor ELK-1, which responds even more dramatically to EGF than does the ER. As shown in Fig. 4B, *left*, each of these reagents nearly abolished the EGF activation of a GAL-Elk1 fusion protein. PD 98059, a specific inhibitor of MEK activation (69), also blocked EGF activation of GAL-Elk1. Thus, these inhibitors work effectively to block transcription mediated by the ERK pathway in these transfected cells. In contrast, only modest (20–40%, shown) decreases in v-Src effects on GAL-ER (A/B) were detected. Even high levels of transiently transfected expression vectors for each dominant negative protein produced no more than 30–40% inhibition of Src action (data not shown). Similar modest inhibition was obtained with PD 98059. These results indicate that Src potentiation of ER AF-1 is only partly mediated by the Ras/Raf/MEK/ERK pathway and suggest that another pathway must also be involved.

Because the Rac-MEKK-JNKK-JNK pathway is also activated by v-Src, we then asked whether this pathway might mediate v-Src effects upon the ER. Dominant negative Rac(S17N) or MEKK1(K432M), strongly inhibited v-Src activation of ER AF-1 (Fig. 4C). In a separate experiment a vector for dominant negative JNKK4(K116R) eliminated the induction mediated by short-term exposure to v-Src expression or stimulation of the endogenous Src/JNK pathway treatment with the cytokine tumor necrosis factor-α (TNFα) (Fig. 4D). These observations indicate that the route from Src to AF-1 proceeds through both the Ras-Raf-MEK and the Rac-MEKK-JNKK pathways and that the ERK and JNK kinases are the true effectors of Src action.

The role of the ERK and JNK kinases was then confirmed by examining the effect of elevated expression of these kinases on Src action. Overexpressed JNK1 and ERK2 synergized with v-Src to activate ER AF-1 (Fig. 4E). Neither JNK nor ERK was sufficient to enhance AF-1 activity in the absence of v-Src, suggesting that both required inputs from upstream kinase cascades to enhance AF-1 activity. In contrast, overexpression of another MAP kinase, p38, which is also activated by JNKK1 (70), inhibited, rather than synergized with, the v-Src activation of ER AF-1. While the mechanism of this dominant negative effect of p38 is unknown, the fact that p38 acts as a dominant negative indicates that p38 does not mediate Src activation of AF-1. In summary, these studies indicate that the route from Src to AF-1 proceeds through both a Ras-Raf-MEK-ERK and a Rac-MEKK-JNKK-JNK pathway.

Src Potentiates AF-1 Both by ERK-Mediated Phosphorylation of S118 and by a JNK-Mediated, S118 Independent, Pathway

As noted previously, activation of the Ras-Raf-MEK-ERK pathway leads to phosphorylation of ER S118 by ERKs. We therefore investigated whether S118, or the nearby S104 and S106, played similar roles in Src activation of AF-1. We first examined the effect of S118 mutations to glutamic acid (E), which mimics the negative charge of phosphorylated serine and allows stronger ER AF-1 binding to p68 RNA helicase (36, 41), or arginine (R), which blocks S118 phosphorylation and shows robust inhibition of ER AF-1 activity (71). As expected, ERS118E showed a slight increase in tamoxifen response relative to ER, and ERS118R showed no tamoxifen response (Fig. 5A). In parallel, transfected v-Src gave a much stronger enhancement of tamoxifen response, suggesting that the increased negative charge of the ER-S118E mutant was insufficient to mimic the enhancement of AF-1 activity that is obtained with v-Src. Furthermore, ERS118E-dependent tamoxifen responses were still strongly enhanced by v-Src, and ERS118R only reduced the overall level

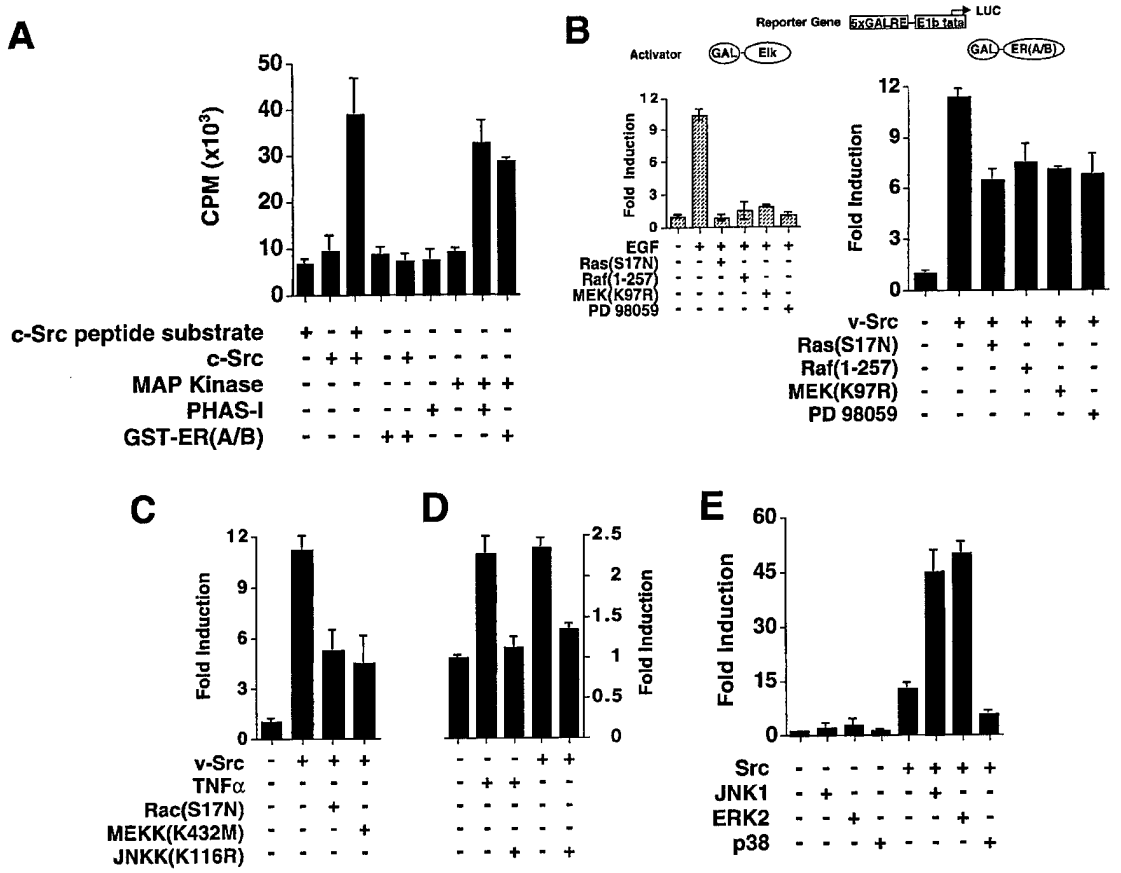

Fig. 4. v-Src Activates AF-1 through ERK and JNK MAP Kinases

A, The ER A/B domain is a target for ERK but not Src kinase. Assay of kinase activity using purified Src and ERK kinases, a peptide Src substrate (amino acids 6–20 of cdc-2), PHAS-I (a peptide ERK substrate), and purified GST-ER(A/B) fusion protein, as indicated. B, v-Src action on AF-1 is partly blocked by dominant negative Ras [Ras(S17N)], Raf1 [Raf(1–257)], MEK [MEK(K97R)], or the PD 98059 compound that blocks activation of ERK MAP kinases. Induction of a GALRE reporter gene activated with GAL-ER(A/B) and v-Src in the presence of the indicated dominant negative or drug inhibitor. *Inset on right* shows the expected complete blockade of EGF action on Elk-1 by the dominant negatives and PD compound. C, v-Src action on AF-1 is also blocked by dominant negative Rac [Rac(S17N)] or MEKK [MEKK(K432M)]. An assay similar to B of reporter gene response to Src in the presence of the indicated dominant negative inhibitor. D, TNFα and Src action on AF-1 is blocked by dominant negative JNKK(K116R). Induction of the GALRE reporter gene activated by GAL-ER(A/B) in HeLa cells cotransfected with dominant negative JNKK [JNKK(K116R)] as indicated. After 20 h incubation, the cells were treated with TNFα for 1 h or transfected with v-Src expression vector, as indicated, and then incubated for 6 h before assay. E, ERK2 and JNK1, but not p38, cooperate with Src to activate AF-1. Induction of the GALRE reporter gene activated by GAL-ER(A/B) in the presence of activated Src and elevated ERK2, JNK1, or p38 as indicated.

of tamoxifen response in the presence of v-Src by about 50%. v-Src also potentiated the tamoxifen responses that were obtained in the presence of an ERS118A mutant (data not shown). Together, these results suggest that v-Src potentiation of AF-1 activity is partially insensitive to mutation of S118. Mutation of S118 to alanine also decreased the ability of v-Src to potentiate GAL-ER(A/B) by 20 to 40% (Fig. 5B and data not shown). There was no further decrease in AF-1 activity when all three serines were mutated to alanine (S104, 106, 118A). Thus, v-Src enhancement of isolated AF-1 is also partially insensitive to mutation of the AF-1 phosphorylation sites.

It is noteworthy that Src has a major effect on AF-1, even in the presence of the triple serine mutation. To test whether this effect is mediated by the non-ERK (that is, JNK) part of the Src-AF-1 pathway, we examined Src activation of wild-type and triple mutant GAL-ER(A/B) in the presence of PD 98059. This inhibitor reduced Src potentiation of AF-1 activity by about 50%, as did the mutation of the three serines (Fig. 5C). However, PD 98059 had no effect on the residual Src activation of the triple serine mutant. This result indicates that the portion of the Src effect that is independent of S118 phosphorylation is also ERK independent. Because JNK activation is not blocked by PD

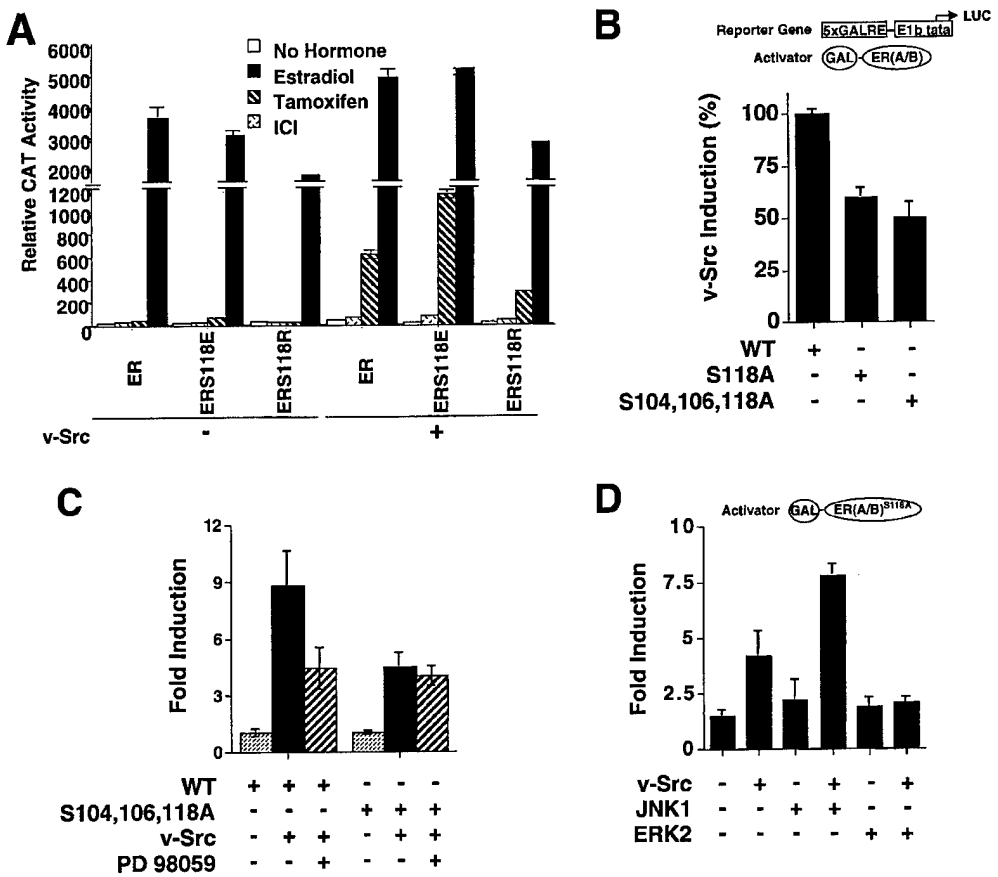

Fig. 5. Src-Activated ERKs, but not JNKs, Require S118 in the A/B Domain to Activate AF- 1

A, Src potentiation of AF-1 is in part independent of S118. Induction of the ERE responsive reporter gene activated by ER, or ERS118E and ERS118R, with and without transfected v-Src. B, Induction of the GALRE reporter gene activated by GAL-ER(A/B) or the indicated mutant thereof, with v-Src. v-Src induction of wild-type GAL-ER(A/B) was set as 100%. C, Src activation of AF-1 that is independent of S118 is resistant to PD 98059, the ERK pathway inhibitor. Src effects are shown as fold induction of reporter gene activity. D, Src potentiation on ER(A/B) with mutations in S118 serine cluster cooperates with JNK but not ERK kinases. Induction of the GALRE reporter gene activated with GAL-ER(A/B) S118, v-Src, and vectors for JNK1 or ERK2, as indicated.

98059 (Ref. 69 and data not shown), we infer that the serine-independent portion of AF-1 activation is likely to be the portion that is mediated by JNKs.

To examine the role of JNKs more directly, we examined the ability of Src and overexpressed JNK to cooperate in AF-1 activation. As shown in Fig. 5D, v-Src and JNK cooperated to enhance the activity of GAL-A/B S118A mutant even more strongly than v-Src alone. Thus, the Src-JNK pathway enhances AF-1 activity in a manner that is independent of phosphorylation at S118. Surprisingly, ERK1 now specifically inhibited v-Src action at the GAL-A/B S118A mutant, much as p38 inhibited v-Src action upon wild-type AF-1 (Fig. 4E). Again, we do not have a ready explanation for this effect but speculate that transfected ERKs could inhibit other second messenger inputs to AF-1 and that this effect is only detectable when their stimulatory effect upon ER AF-1 activity is abolished. Nonetheless, taken together, our results suggest that v-Src enhances AF-1 activity via two independent pathways, one that involves ERKs and targets the AF-1 serine cluster and one that involves JNKs and is independent of the serine cluster.

Src Activates Two Separate Subdomains of AF-1 That Show Differential Sensitivity to Inhibitors of MAP Kinases

AF-1 is complex, and it has been suggested that AF-1 is comprised of independent subdomains (39, 57, 72). We therefore tested some of these candidate subdomains for response to Src. We transfected expression vectors for ERs with specific deletions of the N-terminal (AB) domain into HeLa cells and examined whether they would elicit a tamoxifen response from the ERE-chloramphenicol acetyltransferase (CAT) reporter gene in the presence or absence of cotransfected v-Src (Fig. 6A). As expected, v-Src enhanced the tamoxifen response obtained with wild-type ER by about 8-fold and showed no effect upon an ER trun-

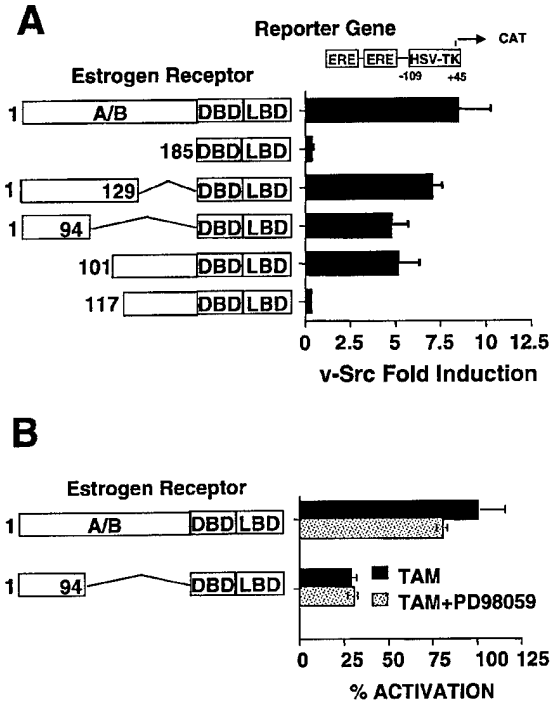

Fig. 6. Src Potentiates Transcription from Two Separable Subdomains of AF-1

A, Fold induction that v-Src elicits from the ERE reporter gene activated by the indicated ER derivative in the presence of tamoxifen. B, The sensitivity of v-Src enhancement of tamoxifen response to PD 98059 was determined in the presence of the indicated ER derivative in the presence of tamoxifen. All values (after subtraction of backgrounds) were normalized to that obtained in the presence of fully wild-type ER, which was set at 100%.

cation that lacked the A/B domain (ΔAB, 185). Transfected v-Src also enhanced the tamoxifen response obtained with an ER truncation that contained amino acids 1–129 by 7-fold. This truncation contains all of the ER sequences that are required for AF-1 activity in HeLa cells (57), so this result underscores the notion that v-Src acts upon AF-1. Transfected v-Src enhanced the tamoxifen responses obtained with ERs bearing either the N-terminal (amino acids 1–94) or C-terminal (amino acids 101–185) AF-1 subdomains by about 5- fold. Furthermore, Src responsiveness of the C-terminal AF-1 subdomain was lost when the region from 101 to 117, which contains the serine cluster, was deleted (117). Together, these observations indicate that v-Src targets two separate subdomains in AF-1. One lies between amino acids 1–94, and a second lies between amino acids 101–129, which overlaps the serine cluster. Although the role of the serine cluster as a target of second messengers is well established, this is the first direct indication that the N-terminal region of AF-1 (amino acids 1–94) contributes to second messenger stimulation of AF-1 activity.

Control transfections revealed that PD 98059 completely failed to inhibit the activity of the ER truncation that only contained the N-terminal AF-1 subdomain (amino acids 1–94, Fig. 6B). Thus, v-Src action upon the N-terminal AF-1 subdomain does not proceed through MAP kinase activation, which implies that v-Src action upon this region proceeds through JNK kinases.

Activated JNKs Do Not Phosphorylate the AF-1 Domain, Suggesting an Indirect Mode of Action

The above studies indicate that Src activates AF-1 through at least two pathways, one of which is mediated through JNK and does not require S118. To investigate whether JNK might phosphorylate another site within AF-1, we tested the ability of JNKs extracted from cells that had been treated with TNFα, the proinflammatory cytokine and activator of Src/JNK (70), for their ability to phosphorylate the AF-1 domain *in vitro*. Activated JNKs were able to efficiently phosphorylate a c-Jun substrate *in vitro* (Fig. 7, *top*) but were unable to phosphorylate an AF-1 substrate, even though this substrate had been efficiently phosphorylated by activated ERK as shown earlier (Fig. 4A). We conclude that activated JNKs do not directly phosphorylate the ER AF-1 domain.

Src Can Potentiate AF-1 in Conditions Where AF-1 Is Mediated by the GRIP1/CBP Complex

Our results show that Src stimulates ER AF-1 activity through JNK kinases and that the JNKs do not phosphorylate the AF-1 domain. This suggests that the action of the JNK pathway on AF-1 may be mediated by phosphorylation of another protein that mediates ER activity. Basal AF-1 activity is mediated by a complex of a p160 such as GRIP1, along with p300/CBP (24). The essential contact for this action is between the N-terminal subdomain of AF-1 and the C-terminal

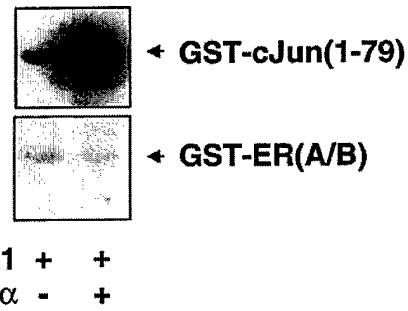

Fig. 7. ER(A/B) Is Not Phosphorylated by JNK1 *in Vitro*

GST-cJun or GST ER(A/B) was *in vitro* phosphorylated by immunopurified JNK1 kinase extracted from HeLa cells treated with TNFα (a Src/JNK activator) and analyzed on 10% SDS-PAGE with autoradiography. Control experiments (see Fig. 3) indicate that GST-ER(A/B) is a good substrate for ERKs.

domain of GRIP1. We therefore asked whether v-Src enhanced AF-1 under conditions in which AF-1 is itself enhanced by overexpression of GRIP1 and CBP.

We first cotransfected expression vectors for GRIP1 or CBP, along with vectors for GAL-AF-1 and v-Src, and asked whether the coactivators would potentiate v-Src action on the GALRE:luc reporter gene. Overexpression of GRIP1 further enhanced v-Src potentiation of ER AF-1 (7- to 9-fold, Fig. 8A, *left*). CBP also slightly increased Src effects on AF-1, similar to its action on basal AF-1 (24). Thus, v-Src synergizes with overexpressed GRIP1 and CBP, suggesting that Src activation of AF-1 can occur when AF-1 activity is mediated by contacts with GRIP1 and CBP. We further examined whether the S118-independent and PD 98059-resistant component of Src action also synergizes with GRIP1. GRIP1 synergized with v-Src to activate GAL-AF-1 with the triple serine mutation S104, 106, 118 A (Fig. 8A, *right*) both with and without PD 98059. We conclude that the S118-independent and PD-resistant component of the Src pathway of AF-1 potentiation is active when AF-1 is mediated by the p160 coactivator GRIP1.

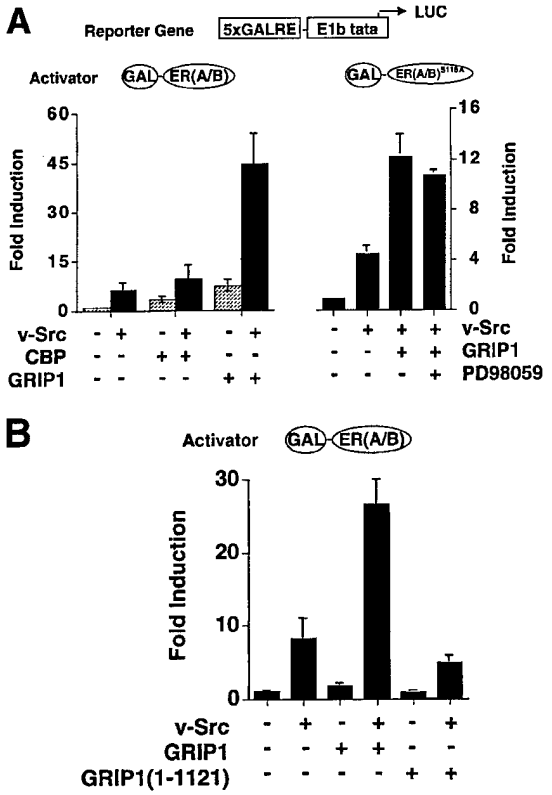

Fig. 8. Src/JNK Potentiates ER AF-1 Mediated by GRIP1 and CBP

A, Elevated GRIP1 or CBP cooperate with Src in activation of AF-1. *Left panel* shows induction of the GALRE reporter gene activated with ER-GAL(A/B) and the effects of transfected v-Src GRIP1 or CBP as indicated. *Right panel* shows the substantial effects of GRIP1 on v-Src potentiation of the triple serine ER-AF-1 mutant even in the presence of PD 98059 as indicated. B, A mutant of GRIP1 deficient for mediating basal AF-1 acts as a partial dominant negative for Src action on AF-1. Response of the reporter gene to v-Src in the presence of elevated amounts of the indicated derivative of GRIP1. GRIP1(1–1,121) is missing amino acids 1,122–1,462 and does not bind to ER(A/B) or mediate AF-1 action in the absence of Src. C, CBP and GRIP1 activation functions are potentiated by Src. Activity of the GALRE:luciferase reporter gene activated by GAL4 fusions to CBP or GRIP1 as indicated and cotransfected with expression vector for v-Src.

As noted above, both AF-1 and AF-2 recruit the p160-CBP complex, but the two ER domains contact different surfaces on the coactivators. AF-1 contacts the C-terminal domain of GRIP1 (24), whereas AF-2 contacts one of the multiple p160 NR Boxes, which are located in the middle portion of GRIP1 (13, 26–29). Thus, deletion of the C terminus of GRIP1 prevents AF-1 activity without affecting AF-2. To test whether AF-1/GRIP1 contacts were needed for v-Src enhancement of AF-1, we overexpressed a C-terminal deletion of GRIP1 (1–1,121 aa), which lacks the site for AF-1 binding (24). The GRIP1 mutant missing the C terminus failed to cooperate with v-Src to enhance AF-1 and reduced activation by v-Src (Fig. 8B). Similar observations were also obtained in the presence of tamoxifen-activated ER (data not shown). Thus, the C-terminus-deleted GRIP1 has a dominant negative effect on Src activation of AF-1. Taken together, these observations confirm that Src can potentiate AF-1 under conditions where AF-1 activity is mediated by contacts with the GRIP1 C terminus. It may also indicate that Src activation of AF-1, like basal AF-1 activity, requires the GRIP1/CBP complex or its functional equivalent.

Src Potentiates CBP and GRIP1 Activation Functions

The above studies suggest that Src potentiation of ER AF-1 is compatible with circumstances in which AF-1 activity is mediated by contacts with GRIP1 and CBP, but the studies are neutral as to whether Src affects the activities of GRIP1 and CBP or some other target. To gain insight into this latter question, we investigated how Src affected the transcriptional activation functions of CBP and GRIP1. GRIP1 or CBP fused to the DNA binding domain of GAL4 activate transcription when they are tethered to the GALRE reporter gene (Fig. 8C). Cotransfection of v-Src greatly increased both the transcriptional activity of CBP and GRIP1. We conclude that v-Src has the potential to change the activities of the GRIP1/CBP coactivator complex. Below we discuss the possibility that this complex may be one of the candidates for a Src/JNK target (see *Discussion*).

DISCUSSION

We were encouraged to investigate whether elevated Src potentiates ER action by two observations. First, in addition to estrogen, ER activity is also stimulated by

signaling cascades initiated at tyrosine kinases. Second, Src tyrosine kinase activity is often elevated in breast cancers, whose proliferation is stimulated by ER activation. Our studies indicate that expression of constitutively active forms of Src or cell stimulation with the cytokine TNFα, both of which lead to JNK activation (56,73), enhances activation of reporter gene expression by the estrogen-ER complex and powerfully enhances the otherwise weak regulation by the tamoxifen-ER complex. These effects are observed both in transiently transfected HeLa cells and in MCF-7 breast cells that express endogenous ERs. Src action upon the ER is mediated through a robust activation of the ER AF-1 function. This is seen most easily when the AF-1 region is removed from the remainder of the ER and fused to a heterologous DNA binding domain from the yeast GAL4 protein. Src action did not require the integrity of tyrosine 537 in the ER-LBD, which has been reported to be directly phosphorylated by Src (61–63). Src tyrosine kinase activity is required for its action upon AF-1, as mutations and drugs that inactivate the kinase block the ability of Src to stimulate ER activity. Thus, taken together, our results suggest that elevated Src kinase activity results in elevated ER AF-1 activity.

The mechanism of Src enhancement of AF-1 activity is unusual. Previous studies have demonstrated that growth factors enhance AF-1 activity via a signal transduction pathway that is mediated by Ras, Raf, MEK, and the ERK kinases. We therefore expected that Src stimulation of ER activity might proceed through a similar pathway. However, transfection of dominant negative Ras, Raf1, and MEK mutants, or treatment of cells with PD 98059, which blocks Raf1 inputs to MEK, only partially inhibited Src potentiation of AF-1. This suggested that Src also enhanced AF-1 activity via a second pathway and, indeed, dominant negative versions of Rac, MEKK, and JNKK also inhibited Src enhancement of AF-1 activity. Src cooperates both with overexpressed ERK and overexpressed JNK to generate even higher AF-1 activity. Thus, the Src pathway leading to potentiation of AF-1 proceeds through JNKs and through ERKs.

Although the pathway from Src to enhancement of AF-1 activity clearly runs through Rac/MEKK/JNKK and JNKs, it is unclear how the JNKs bring about the enhancement of AF-1 activity. One possibility is that the JNKs directly phosphorylate the AF-1 domain and thereby modify its function, as previously shown for transcription factors c-jun (74, 75) and Elk-1 (76). Our *in vitro* studies weigh against this possibility, as they indicate that JNKs fail to bind AF-1 (data not shown) and that they also fail to phosphorylate the ER A/B domain *in vitro*. Although it is possible that these failures reflect a requirement for an accessory protein not supplied *in vitro*, it is most likely an indication that the JNKs cannot efficiently phosphorylate the AF-1 domain, as activated JNKs require no accessory proteins to phosphorylate other substrates (75, 77, 78). Our *in vivo* studies also suggest that JNK action is independent of direct ER phosphorylation. The major site of phosphorylation in the AF-1 region is S118, and mutation of this residue to alanine blocks AF-1 phosphorylation and enhancement of AF-1 activity by EGF, activated Ras, and other activators of ERK kinases. JNKs do not activate AF-1 via phosphorylation at S118. Replacement of S118 with alanine only partly reduces Src stimulation of AF-1 activity, and the residual activation is mediated through an ERK-independent pathway. We infer that this residual action is due to Src-activated JNK and this has been confirmed with overexpressed JNKs. In addition, the Src-to-JNK cascade targets at least two subdomains within AF-1, from amino acids 1–94, and 101–129. The first of these domains contains no potential sites for JNK phosphorylation. Furthermore, replacement of the serine cluster, including the MAP kinase phosphorylation site (S118), has no effect on the response of AF-1 to JNK activation.

How does Src-activated JNK enhance AF-1? One attractive possibility is that activated JNKs target a protein that, itself, affects AF-1 action. Recent studies indicate that AF-1 works by recruiting p160/CBP coactivators by means of a direct contact between AF-1 and the C terminus of the p160 (24). We have confirmed that Src enhancement of AF-1 activity can also occur in the presence of overexpressed GRIP1 and CBP, and that this enhancement requires the C terminus of GRIP1. Thus, one possibility is that the AF-1 mediating activities of the p160/CBP complex are the target for JNK. Accordingly, v-Src was able to enhance the transcriptional activity of both GRIP1 and CBP when they were directly tethered to DNA. One obvious question is that if, indeed, v-Src does enhance the activity of the GRIP1/CBP complex, and given that both AF-1 and AF-2 work by binding a GRIP1/CBP complex, then why would v-Src preferentially enhance AF-1 activity? We have previously shown that the AF-1 and AF-2 functions of different nuclear receptors both bind to p160s, but bind to different surfaces and require different transcriptional outputs (24,79). Thus, v-Src could preferentially affect AF-1 activity by preferentially affecting a subset of p160 transcriptional inputs or outputs that are required for AF-1 action.

We stress that the sole positive evidence for Src/JNK targeting of the coactivator complex is that Src potentiates the transcriptional activation functions of both GRIP1 and CBP when they are fused to GAL4 and tethered to a promoter. This experiment is only suggestive. While JNKs might phosphorylate GRIP1 itself, or another component of the complex (Fig. 9), there are other, equally likely, possibilities. For example, JNKs might phosphorylate and modify the activities of corepressors that are suspected of modulating AF-1 action. Exploratory studies are underway to examine these and other possibilities.

Finally, we speculate that Src enhancement of AF-1 may have consequences for cellular responses to estrogen. Breast cancer samples and cell lines almost invariably have elevated Src tyrosine kinase activity

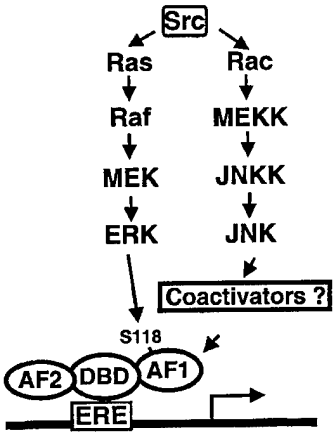

Fig. 9. Pathways of v-Src Stimulation of ER(A/B) Transcriptional Activity

One pathway leads from Src through Ras, Raf, MEK, and ERKs and results in phosphorylation of the ER(A/B) on S118. The second pathway leads to Rac, MEKK, JNKK, and JNKs. JNKs do not phosphorylate S118. Among many possibilities JNKs may target coactivators that mediate AF-1 activity. This is indicated by an *arrow* from JNK to coactivators. That it is merely a possibility is indicated by the *question mark*.

(50). Some of those tumors and cell lines also express ER and are stimulated to grow by estrogens. In such cases, ER is believed to enhance proliferation by enhancing the expression of target genes encoding regulators of proliferation. We showed here that overexpression of v-Src leads to increases in ER transcriptional activity in breast cells, and that this effect occurs in the presence of estradiol, the absence of hormone and, most strikingly, in the presence of tamoxifen. Thus, increases in Src kinase activity could, via stimulation of ER transcriptional potency, enhance the ability of ER to induce cellular proliferation in breast cancer and breast cancer cell lines. Moreover, increased Src kinase activity could also lead to increased tamoxifen agonist activity, which might play a role in the development of tamoxifen resistance. We note that the tyrosine kinase inhibitors genistein and Herbimycin A inhibit cellular proliferation in response to estrogen (80–83). Both of these inhibitors block Src activity in breast cancer cells and also inhibit Src effects upon ER AF-1. Thus, it is conceivable that both drugs could inhibit estrogen-induced proliferation via inhibition of Src effects upon the ER. Taken together, our results suggest that it is important to investigate the relationship of Src activity and ER action in breast cancer and during tumor progression.

MATERIALS AND METHODS

Mammalian Reporter Genes and Expression Vectors

EREII-HSV-TK-CAT, a reporter gene containing two vitellogenin EREs upstream of the herpes simplex virus TR proximal promoter (−109/+45), has been previously described (84). The GAL4 responsive reporter gene GK1 contains five GAL4 response elements upstream of a minimal adenovirus E1b promoter that has been previously described (85).

ER expression vectors pSG5-ER, pSG5-ERG400V have been described previously (86). pSG5-ERΔ(A/B), and Δ1–100, Δ1–116, Δ130–184, and Δ95–184 have been described previously (57). pSG5-GAL4, -GAL4-ER(A/B), -GAL4-ER-(LBD), -GAL4-VP16 expression vectors have been described (57, 87). The pSG5-ERY537R, Y537S mutants were obtained from Dr. G. Greene, University of Chicago. The pCMV-ERY537F mutant was obtained form Dr. B. Katzenellenbogen, University of Illinois. The pSG5-GAL4-ER(A/B) mutants in which each the phosphorylation sites at serine (S) 104, S106, S118, were mutated to alanine (A) were created by synthesizing double-stranded oligonucleotides that encode the mutant sequence and using Quick Change Site-directed Mutagenesis Kits (Stratagene, La Jolla, CA). The mutated sequences were verified by DNA sequencing using Sequenase Kits (Stratagene). The GAL-4-ER(A/B) mutant containing the triple phosphorylation site mutation (S104, 106, 118A) was made by multiple rounds of mutagenesis.

Many signal transduction molecule expression vectors were kindly provided as follows: pCMV-v-Src (Dr. M. Bishop, University of California, San Francisco, CA); pCMV-c-SrcRF(K295R,Y527F) and pCMV-c-Src (Dr. Joan Brugge, Ariad Pharmaceuticals, Inc., Cambridge, MA); Constitutively activated pCMV-c-Src (Y527F) (Dr. Tony Hunter, Salk Institute for Biological Studies, La Jolla, CA); dominant negative pcDNA3-Rac1(S17N) and -Raf(1–257) (Dr. H. Goldberg, University of Toronto, Toronto, Ontario, Canada); pCMV-Flag-p38 (Dr. Roger Davis, University of Massachusetts Medical Center, Boston, MA). Dominant negative Ras(S17N), MEK(K97R), pSRα-JNKK(K116R), MEKK(K432M), HA-JNK1, and HA-ERK2 have been described previously (56). Coactivator expression vector pCMV-CBP was a gift from Dr. M. Rosenfeld (University of California San Diego, La Jolla, CA). pSG5-GRIP1 has been previously described (29).

Cell Culture and Transfection

HeLa cells were maintained and transfected as previously described (86). Briefly, around 70% confluent HeLa cells were transfected with 5 μg of $(ERE)_2$-TK-CAT or 5x GAL4-Luc reporter gene, 1 μg β-galactosidase plasmid, 1 μg ER expression vector, and other coactivator and signal molecule expression vectors (2 μg) as indicated in the figure legends. After 20 h incubation, cells were lysed and CAT, LUC, and β-galactosidase assays were performed using standard methods. The β-galactosidase activity was used to correct the variations of transfection efficiency in CAT and LUC activities. The hormones (10 nM estradiol, ICI, and 5 μM tamoxifen) and kinase inhibitors [300 μM genistein (Sigma, St. Louis, MO), or 1 μM herbimycin A (Sigma), and 100 μm PD 98059 (Calbiochem)] were added immediately after transfection. CAT and LUC activities represented the averages from triplicate wells with less than 20% deviation. Experiments were repeated at least three times.

Western Blots

HeLa cells were transfected with ER and v-Src expression vectors, or empty control vectors, and allowed to stand overnight. Half were prepared for Western blot analysis, and the remaining half was used for standard CAT and β-galactosidase assays. The following day, the transfected cells were washed in cold PBS and treated with 1 ml of luciferase lysis buffer on ice for 5 min. The lysate was scraped off the dish, transferred to Eppendorf (Madison, WI) tubes, and pelleted in an Eppendorf microfuge for 15 min at 4 C. Protein contents were determined and 15 μg of cell proteins were separated on a 10% SDS-polyacrylamide gel and transferred to a pre-

moistened Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc. Hercules CA), overnight at 90 mA, 30V, using a standard transfer apparatus. After transfer, the membrane was incubated at room temperature in 5% nonfat milk in PBS-T (1×PBS, 0.1% Tween-20) for 1 h, and washed twice in PBS-T for 10 min. The primary anti-ER antibody (HC-20, Santa Cruz Biotechnology, Inc.,, Santa Cruz, CA) was diluted 1:2,000 in PBS-T and incubated with the membrane for 1 h, followed by PBS-T washes, once for 15 min and then twice for 5 min. The membrane was then incubated for 45 min with horseradish peroxidase-conjugated antirabbit IgG (Santa Cruz Biotechnology, Inc.) diluted 1:2,000 in PBS-T, followed by PBS-T washes, once for 15 min and four times for 5 min in PBS-T. After the last wash, the membrane was developed according to manufacturer's instructions with a standard ECL kit (Amersham Pharmacia Biotech, Arlington Heights, IL), covered with Saran wrap and exposed to x-ray film.

GST-Fusion Proteins

GST-ER(A/B) and GST-cJun (amino acids 1–79, from Dr. A. DeFranco, UCSF) fusion protein were prepared as previously described (86). Briefly, bacteria (500 ml LB media) expressing the fusion proteins were resuspended in 15 ml of TST buffer (0.5 M Tris, 1.5 M NaCl, 0.5% Tween 20, pH 7.5) and sonicated mildly for 2–3 min in ice. The debris was pelleted at 12,000 rpm for 1 h in an ss34 rotor. The supernatant was rotated gently for 2 h in a cold room with 0.5 ml of glutathione sepharose 4B beads that had been prewashed with 5–10 vol of TST buffer. GST-fusion protein beads were washed with 10–20 vol PBS 0.01% Nonidet P-40 and resuspended in 1:1 vol of 20 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors, pH 7.9, for storage at 4 C until use.

In Vitro Kinase Assay

Src, MAP, and JNK kinase assays were carried out following the manufacturer's protocol (Upstate Biotechnology, Inc., Lake Placid, NY) with purified Src, MAP, and JNK kinases, as provided. Briefly, purified Src, MAP, and JNK kinases were mixed with the corresponding substrates or GST-ER(A/B), the reaction was started by adding the corresponding reaction buffer containing (γ-^{32}P)ATP, and then mixed gently and incubated at 30 C for 15–30 min. After addition of 40% trichloroacetic acid or 2× SDS-PAGE loading buffer to stop the reaction, the phosphorylated kinase substrates and GST-ER(A/B) were detected by liquid scintillation counter or autoradiography of 10–12% SDS-PAGE.

Acknowledgments

We thank David Stokoe for helpful comments on the manuscript.

Received April 7, 1999. Re-revision received October 5, 2000. Accepted October 9, 2000.

Address requests for reprints to: Peter Kushner, Metabolic Research Unit 1119 HSW, University of California, San Francisco, CA 94143-0540. E-mail: kushner@itsa.ucsf.edu.

This work was supported by Public Health Service Grants DK-51083 and CA-80210 (to P.J.K.) and by grants from the California Breast Cancer Research Program (to P.J.K. and M.K.).

Peter Kushner wishes to inform readers that he has significant financial holdings in and is a consultant to KaroBio AB, a pharmaceutical company with interests in nuclear receptors.

REFERENCES

1. Parker MG 1998 Transcriptional activation by oestrogen receptors. Biochem Soc Symp 63:45–50
2. Parker MG 1996 Antiestrogen-estrogen receptor interactions. Cancer Treat Res 83:213–224
3. Berry M, Metzger D, Chambon P 1990 Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. EMBO J 9:2811–2818
4. Metzger D, Berry M, Ali S, Chambon P 1995 Effect of antagonists on DNA binding properties of the human estrogen receptor *in vitro* and *in vivo*. Mol Endocrinol 9:579–591
5. McDonnell DP, Clemm DL, Hermann T, Goldman ME, Pike JW 1995 Analysis of estrogen receptor function *in vitro* reveals three distinct classes of antiestrogens. Mol Endocrinol 9:659–669
6. Glass CK, Rose DW, Rosenfeld MG 1997 Nuclear receptor coactivators. Curr Opin Cell Biol 9:222–232
7. Shibata H, Spencer TE, Onate SA, Jenster G, Tsai SY, Tsai MJ, O'Malley BW 1997 Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. Recent Prog Horm Res 52:141–164
8. Onate SA, Tsai SY, Tsai MJ, O'Malley BW 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270: 1354–1357
9. Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Gloss B, Lin SC, Heyman RA, Rose DW, Glass CK, Rosenfeld MG 1996 A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell 85:403–414
10. Hong H, Kohli K, Trivedi A, Johnson DL, Stallcup MR 1996 GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. Proc Natl Acad Sci USA 93:4948–4952
11. Voegel JJ, Heine MJS, Zechel C, Chambon P, Gronemeyer H 1996 TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. EMBO J 15:3667–3675
12. Hong H, Kohli K, Garabedian M, Stallcup M 1997 GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. Mol Cell Biol 17:2735–2744
13. Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfeld MG 1997 The transcriptional coactivator p/CIP binds CBP and mediates nuclear-receptor function. Nature 387:677–684
14. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM, Meltzer PS 1997 AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277:965–968
15. Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, Privalsky ML, Nakatani Y, Evans RM 1997 Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 90:569–580
16. Li H, Gomes PJ, Chen JD 1997 RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. Proc Natl Acad Sci USA 94:8479–8484
17. Takeshita A, Cardona GR, Koibuchi N, Suen CS, Chin WW 1997 TRAM-1, a novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator-1. J Biol Chem 272: 27629–27634
18. Chakravarti D, LaMorte V, Nelson M, Nakajima T, Schulman I, Juguilon H, Montminy M, Evans R 1996 Role of CBP/P300 in nuclear receptor signalling. Nature 383: 99–103

19. Hanstein B, Eckner R, DiRenzo J, Halachmi S, Liu H, Searcy B, Kurokawa R, Brown M 1996 p300 is a component of an estrogen receptor coactivator complex. Proc Natl Acad Sci USA 93:11540–11545
20. Yao TP, Ku G, Zhou N, Scully R, Livingston DM 1996 The nuclear hormone receptor coactivator SRC-1 is a specific target of p300. Proc Natl Acad Sci USA 93: 10626–10631
21. Yang XJ, Ogryzko VV, Nishikawa J, Howard BH, Nakatani Y 1996 A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature 382: 319–324
22. Scolnick DM, Chehab NH, Stavridi ES, Lien MC, Caruso L, Moran E, Berger SL, Halazonetis TD 1997 CREB-binding protein and p300/CBP-associated factor are transcriptional coactivators of the p53 tumor suppressor protein. Cancer Res 57:3693–3696
23. Korzus E, Torchia J, Rose DW, Xu L, Kurokawa R, McInerney EM, Mullen TM, Glass CK, Rosenfeld MG 1998 Transcription factor-specific requirements for coactivators and their acetyltransferase functions. Science 279: 703–707
24. Webb P, Nguyen P, Shinsako J, Anderson CM, Nguyen MP, McKinerney E, Katzenellenbogen BS, Stallcup M, Kushner PJ 1998 Estrogen receptor activation function 1 works by binding p160 coactivator proteins. Mol Endocrinol 12:1605–1618
25. Le DB, Nielsen AL, Garnier JM, Ichinose H, Jeanmougin F, Losson R, Chambon P 1996 A possible involvement of TIF1α and TIF1β in the epigenetic control of transcription by nuclear receptors. EMBO J 15:6701–6715
26. Voegel JJ, Heine MJ, Tini M, Vivat V, Chambon P, Gronemeyer H 1998 The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways. EMBO J 17:507–519
27. Heery DM, Kalkhoven E, Hoare S, Parker MG 1997 A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387:733–736
28. Kalkhoven E, Valentine JE, Heery DM, Parker MG 1998 Isoforms of steroid receptor co-activator 1 differ in their ability to potentiate transcription by the oestrogen receptor. EMBO J 17:232–243
29. Ding XF, Anderson CM, Ma H, Hong H, Uht RM, Kushner PJ, Stallcup MR 1998 Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. Mol Endocrinol 12:302–313
30. Bannister AJ, Kouzarides T 1996 The CBP co-activator is a histone acetyltransferase. Nature 384:641–643
31. Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y 1996 The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 87:953–959
32. Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, McKenna NJ, Onate SA, Tsai SY, Tsai MJ, O'Malley BW 1997 Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 389:194–198
33. Ignar-Trowbridge DM, Pimentel M, Teng CT, Korach KS, McLachlan JA 1995 Cross talk between peptide growth factor and estrogen receptor signaling systems. Environ Health Perspect 7:35–38
34. Ignar-Ttrowbridge DM, Pimentel M, Parker MG, McLachlan JA, Korach KS 1996 Peptide growth factor cross-talk with the estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. Endocrinology 137:1735–1744
35. El TM, Green CD 1997 Two separate mechanisms for ligand-independent activation of the estrogen receptor. Mol Endocrinol 11:928–937
36. Bunone G, Briand PA, Miksicek RJ, Picard D 1996 Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 15:2174–2183
37. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H. Metzger D, Chambon P 1995 Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270:1491–1494
38. Joel PB, Traish AM, Lannigan D 1998 Estradiol-induced phosphorylation of serine 118 in the estrogen receptor is independent of p42/p44 mitogen-activated protein kinase. J Biol Chem 273:13317–13323
39. Ali S, Metzger D, Bornert JM, Chambon P 1993 Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. EMBO J 12:1153–1160
40. Le GP, Montano MM, Schodin DJ, Katzenellenbogen BS 1994 Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. J Biol Chem 269:4458–4466
41. Endoh H, Maruyama K, Masuhiro Y, Kobayashi Y, Goto M, Tai H, Yanagisawa J, Metzger D, Hashimoto S, Kato S 1999 Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor α. Mol Cell Biol 19:5363–5372
42. LeMaistre CF, Meneghetti C, Howes L, Osborne CK 1994 Targeting the EGF receptor in breast cancer treatment. Breast Cancer Res Treat 32:97–103
43. Chrysogelos SA, Dickson RB 1994 EGF receptor expression, regulation, and function in breast cancer. Breast Cancer Res Treat 29:29–40
44. Earp HS, Dawson TL, Li X, Yu H 1995 Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. Breast Cancer Res Treat 35: 115–132
45. Reese DM, Slamon DJ 1997 HER-2/neu signal transduction in human breast and ovarian cancer. Stem Cells 15:1–8
46. Jacobs C, Rubsamen H 1983 Expression of pp60c-src protein kinase in adult and fetal human tissue: high activities in some sarcomas and mammary carcinomas. Cancer Res 43:1696–1702
47. Rosen N, Bolen JB, Schwartz AM, Cohen P, DeSeau V, Israel MA 1986 Analysis of pp60c-src protein kinase activity in human tumor cell lines and tissues. J Biol Chem 261:13754–13759
48. Ottenhoff KA, Rijksen G, van Beurden E, Hennipman A, Michels AA, Staal GE 1992 Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-src oncogene product. Cancer Res 52: 4773–4778
49. Verbeek BS, Vroom TM, Adriaansen SS, Ottenhoff KA, Geertzema JG, Hennipman A, Rijksen G 1996 c-Src protein expression is increased in human breast cancer. An immunohistochemical and biochemical analysis. J Pathol 180:383–388
50. Muthuswamy SK, Muller WJ 1994 Activation of the Src family of tyrosine kinases in mammary tumorigenesis. Adv Cancer Res 64:111–123
51. Brown MT, Cooper JA 1996 Regulation, substrates and functions of src. Biochim Biophys Acta 1287:121–149
52. Parsons JT, Parsons SJ 1997 Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways. Curr Opin Cell Biol 9:187–192
53. Thomas SM, Brugge JS 1997 Cellular functions regulated by Src family kinases. Annu Rev Cell Dev Biol 13:513–609
54. Xie W, Herschman HR 1995 v-src Induces prostaglandin synthase 2 gene expression by activation of the c-Jun N-terminal kinase and the c-Jun transcription factor. J Biol Chem 270:27622–27628

55. Brenner B, Weinmann S, Grassme H, Lang F, Linderkamp O, Gulbins E 1997 L-selectin activates JNK via src-like tyrosine kinases and the small G-protein Rac. Immunology 92:214–219
56. Minden A, Lin A, Claret FX, Abo A, Karin M 1995 Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell 81:1147–1157
57. Metzger D, Ali S, Bornert JM, Chambon P 1995 Characterization of the amino-terminal transcriptional activation function of the human estrogen receptor in animal and yeast cells. J Biol Chem 270:9535–9542
58. Tzukerman MT, Esty A, Santiso MD, Danielian P, Parker MG, Stein RB, Pike JW, McDonnell DP 1994 Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol Endocrinol 8:21–30
59. McDonnell DP, Dana SL, Hoener PA, Lieberman BA, Imhof MO, Stein RB 1995 Cellular mechanisms which distinguish between hormone- and antihormone-activated estrogen receptor. Ann NY Acad Sci 761:121–137
60. Tora L, Mullick A, Metzger D, Ponglikitmongkol M, Park I, Chambon P 1989 The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. EMBO J 8:1981–1986
61. Arnold SF, Vorojeikina DP, Notides AC 1995 Phosphorylation of tyrosine 537 on the human estrogen receptor is required for binding to an estrogen response element. J Biol Chem 270:30205–30212
62. Arnold SF, Melamed M, Vorojeikina DP, Notides AC, Sasson S 1997 Estradiol-binding mechanism and binding capacity of the human estrogen receptor is regulated by tyrosine phosphorylation. Mol Endocrinol 11:48–53
63. Auricchio F, Di Domenico M, Migliaccio A, Castoria G, Bilancio A 1995 The role of estradiol receptor in the proliferative activity of vanadate on MCF-7 cells. Cell Growth Diff 6:105–113
64. White R, Sjšberg M, Kalkhoven E, Parker MG 1997 Ligand-independent activation of the oestrogen receptor by mutation of a conserved tyrosine. EMBO J 16: 1427–1435
65. Weis KE, Ekena K, Thomas JA, Lazennec G, Katzenellenbogen BS 1996 Constitutively active human estrogen receptors containing amino acid substitutions for tyrosine 537 in the receptor protein. Mol Endocrinol 10: 1388–1398
66. Yudt MR, Vorojeikina D, Zhong L, Skafar DF, Sasson S, Gasiewicz TA, Notides AC 1999 Function of estrogen receptor tyrosine 537 in hormone binding, DNA binding, and transactivation. Biochemistry 38:14146–14156
67. Berthois Y, Pourreau SN, Gandilhon P, Mittre H, Tubiana N, Martin PM 1986 Estradiol membrane binding sites on human breast cancer cell lines. Use of a fluorescent estradiol conjugate to demonstrate plasma membrane binding systems. J Steroid Biochem 25:963–972
68. Razandi M, Pedram A, Greene G, Levin E 1999 Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERα and ER β expressed in Chinese hamster ovary cells. Mol Endocrinol 13:307–319
69. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR 1995 PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J Biol Chem 270:27489–27494
70. Lin A, Minden A, Martinetto H, Claret FX, Lange-Carter C, Mercurio F, Johnson GL, Karin M 1995 Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. Science 268:286–290
71. Webb P, Nguyen P, Valentine C, Lopez G, Kwok GR, McInerney E, Katzenellenbogen BS, Enmark E, Gustafsson JA, Nilsson S, Kushner PJ 1999 The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. Mol Endocrinol 13:1672–1685
72. McInerney EM, Katzenellenbogen BS 1996 Different regions in activation function-1 of the human estrogen receptor required for antiestrogen- and estradiol-dependent transcription activation. J Biol Chem 271: 24172–24178
73. Lin A, Smeal T, Binetruy B, Deng T, Chambard JC, Karin M 1993 Control of AP-1 activity by signal transduction cascades. Adv Second Messenger Phosphoprotein Res 28:255–260
74. Smeal T, Binetruy B, Mercola DA, Birrer M, Karin M 1991 Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. Nature 354:494–496
75. Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ 1994 JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 76:1025–1037
76. Cavigelli M, Dolfi F, Claret FX, Karin M 1995 Induction of c-fos expression through JNK-mediated TCF/Elk-1 phosphorylation. EMBO J 14:5957–5964
77. Hibi M, Lin A, Smeal T, Minden A, Karin M 1993 Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev 7:2135–2148
78. Kallunki T, Deng T, Hibi M, Karin M 1996 c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. Cell 87:929–939
79. Ma H, Hong H, Huang SM, Irvine RA, Webb P, Kushner PJ, Coetzee GA, Stallcup MR 1999 Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. Mol Cell Biol 19:6164–6173
80. Clark JW, Santos MA, Stevenson LE, Frackelton AJ 1996 Effects of tyrosine kinase inhibitors on the proliferation of human breast cancer cell lines and proteins important in the ras signaling pathway. Int J Cancer 65:186–191
81. Hoffman R 1995 Potent inhibition of breast cancer cell lines by the isoflavonoid kievitone: comparison with genistein. Biochem Biophys Res Commun 211:600–606
82. Panno ML, Salerno M, Pezzi V, Sisci D, Maggiolini M, Mauro L, Morrone EG, Ando S 1996 Effect of oestradiol and insulin on the proliferative pattern and on oestrogen and progesterone receptor contents in MCF-7 cells. J Cancer Res Clin Oncol 122:745–749
83. Peterson G, Barnes S 1996 Genistein inhibits both estrogen and growth factor-stimulated proliferation of human breast cancer cells. Cell Growth Differ 7:1345–1351
84. Webb P, Lopez GN, Greene GL, Baxter JD, Kushner PJ 1992 The limits of the cellular capacity to mediate an estrogen response. Mol Endocrinol 6:157–167
85. Saatcioglu F, Lopez G, West BL, Zandi E, Feng W, Lu H, Esmaili A, Apriletti JW, Kushner PJ, Baxter JD, Karin M 1997 Mutations in the conserved C-terminal sequence in thyroid hormone receptor dissociate hormone-dependent activation from interference with AP-1 activity. Mol Cell Biol 17:4687–4695
86. Webb P, Lopez GN, Uht RM, Kushner PJ 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol 9:443–456
87. Sadovsky Y, Webb P, Lopez G, Baxter JD, Fitzpatrick PM, Gizang GE, Cavailles V, Parker MG, Kushner PJ 1995 Transcriptional activators differ in their responses to overexpression of TATA-box-binding protein. Mol Cell Biol 15:1554–1563

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 25, Issue of June 22, pp. 22177–22182, 2001
Printed in U.S.A.

Growth Factors Signal to Steroid Receptors through Mitogen-activated Protein Kinase Regulation of p160 Coactivator Activity*

Received for publication, November 28, 2000, and in revised form, March 26, 2001
Published, JBC Papers in Press, April 11, 2001, DOI 10.1074/jbc.M010718200

Gabriela N. Lopez‡, Christoph W. Turck§¶, Fred Schaufele‡, Michael R. Stallcup‖, and Peter J. Kushner‡§**

From the ‡Metabolic Research Unit, §Department of Medicine, and ¶Howard Hughes Medical Institute, University of California, San Francisco, California 94143 and the ‖Department of Pathology, University of Southern California, Los Angeles, California 90033

Promoter-bound steroid receptors activate gene expression by recruiting members of the p160 family of coactivators. Many steroid receptors, most notably the progesterone and estrogen receptors, are regulated both by cognate hormone and independently by growth factors. Here we show that epidermal growth factor regulates the activities of the p160 GRIP1 through the extracellular signal-regulated kinase (ERK) family of mitogen-activated protein kinases. ERKs phosphorylate GRIP1 at a specific site, Ser-736, the integrity of which is required for full growth factor induction of GRIP1 transcriptional activation and coactivator function. We propose that growth factors signal to nuclear receptors in part by targeting the p160 coactivators.

Nuclear receptors such as the estrogen receptor (ER)[1] and progesterone receptor (PR) tether via their DNA binding domain to response elements in the promoter region of target genes and stimulate transcription. To do so the receptors must bind to coactivators that they recruit through transcriptional activation functions, the constitutive AF-1, found in the amino-terminal receptor domain, and the hormone-activated AF-2 in the carboxyl ligand binding domain (LBD) (for review, see Ref. 1). Perhaps the most important of these coactivators is the p160 family, SRC-1 (N-CoA1), GRIP1 (TIF2/N-CoA2), and ACTR (pCIP/AIB1/RAC3). These bind to the LBD only in the presence of cognate hormone, and their binding is blocked by antagonist ligands. The mechanism of binding is now understood in atomic detail and involves the docking of coactivator nuclear receptor boxes, which have the motif LXXLL with a hydrophobic cleft that forms on the surface of the hormone-bound LBD (2–6). The AF-1 domain of the estrogen, androgen, and perhaps other receptors also contacts the p160s but does so through surfaces outside of the nuclear receptor boxes (7, 8).

The p160s are complex proteins with multiple domains (Fig. 1). In addition to the nuclear receptor boxes they have two intrinsic transcriptional activation domains AD1 and AD2, whose activities may be monitored when the coactivators are directly tethered on DNA via fusion to a heterologous DNA binding domain (8, 9). AD1, which is essential for transcriptional mediation by p160s, is coextensive with the binding domain for the CBP/p300 family of coactivators. CBP/p300 complexes with the p160s and synergizes in coactivator function (10, 11). In particular, CBP/p300s contain a potent acetyltransferase activity that can transfer acetate from acetyl-CoA to histones and also to other proteins in the complex on DNA (12–18). AD2 contributes to coactivation by p160s in some circumstances and does so in part by binding CARM1 and other proteins that have histone methyltransferase activity (19). The coactivators are believed to mediate transcriptional activation by remodeling chromatin through their histone modification activities and also by direct effects on the transcriptional complex.

In addition to regulating AF-2, hormones regulate steroid receptors (but not other nuclear receptors) in part by releasing the receptors from inhibitory complexes with heat shock proteins. The activity of steroid receptors is not regulated solely by hormones, however. Growth factors, such as EGF and insulin-like growth factor 1, can have profound and surprising effects on steroid receptors, even in the absence of cognate hormone (for review, see Ref. 20). In the most dramatic examples, EGF activates progesterone receptors almost as well as does hormone (21) and will partly activate ER in the absence of hormone and enhance activity in the presence of hormone (22). This later activity has been traced in part to EGF initiation of a cascade through the ERK family of MAP kinases that ultimately phosphorylates the ER at Ser-118 in the AF-1 region (23–26). Phosphorylation of Ser-118 leads to increased AF-1 activity by increasing binding of a p68 RNA helicase that is or becomes bound to CBP. This Ser-118-dependent link of AF-1 to CBP is in addition to the Ser-118-independent link with GRIP1 and thereby CBP (27).

Although EGF-mediated phosphorylation of steroid receptors underlies part of EGF enhancement, it cannot underlie all of it. In particular, mutation of Ser-118 to glutamate in the ER AF-1 domain, while blocking phosphorylation, nonetheless preserves EGF enhancement of ER action (25). Furthermore, careful mutation of sites of phosphorylation in the progesterone receptor again reveals a potent action of EGF in the absence of direct receptor phosphorylation (21). These studies suggest the existence of a pathway of EGF action that targets a nonrecep-

* This work was supported by United States Public Health Service Grants R01 CA 80210 and R01 DK51083 from the National Institutes of Health and Grant DAMD17-99-1-9110 from the Army Breast Cancer Research Program (to P. J. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Director of, consultant to, and has significant financial interests in KaroBio AB, a Swedish pharmaceutical development company with interests in this area of research. To whom correspondence should be addressed. Tel.: 415-476-6790; E-mail: kushner@itsa.ucsf.edu.

[1] The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; LBD, ligand binding domain; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; HPLC, high pressure liquid chromatography; GFP, green fluorescent protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; JNK, c-Jun NH_2-terminal kinase; GST, glutathione *S*-transferase; luc, luciferase; DBD, DNA binding domain; ERE, estrogen response element; PRE, progesterone response element; wt, wild type; TGF, transforming growth factor; CBP, CREB-binding protein; GRIP1, glucocorticoid receptor interacting protein 1.

tor protein. We have explored the possibility that this unknown target is one of the p160 coactivators, particularly GRIP1. We present evidence that at least part of the signal from EGF to steroid receptor is conducted through the p160 coactivators.

EXPERIMENTAL PROCEDURES

Cell Culture, Transient Transfection, and Luciferase Assay

HeLa cells were transfected by electroporation as previously described (28). Generally transfections included 5 μg of luciferase reporter plasmid, 1.0 μg of Gal-GRIP expression vectors, and 1.0 μg of β-actin-β-galactosidase expression vector for internal control. After electroporation, the cells were treated with vehicle, EGF (25 ng/ml), or TGF-α (15 ng/ml). Luciferase and chloramphenicol acetyltransferase values are the means and standard deviations of triplicate treatments from a single experiment, representative of at least three independent experiments.

In Vitro Kinase Assay

GST-GRIP fragments were expressed in bacteria (HB101) and partially purified by glutathione-Sepharose affinity column. Beads bearing the fusion proteins (1–3 μg of total protein) were subjected to *in vitro* phosphorylation by activated ERK2 according to the instructions of the supplier (Stratagene, La Jolla, CA). The phosphorylated products were extracted from the beads, resolved by 10% polyacrylamide gel electrophoresis, stained with Coomassie Blue to monitor expression, and subjected to autoradiography.

Phosphorylation Site Mapping

Approximately 8.5 μg of total protein on beads were labeled in a 50-μl final reaction volume, extracted from the beads, and purified by 10% SDS-polyacrylamide gel electrophoresis. The gel was fixed, dried, and exposed to x-ray film to visualize the radiolabeled proteins. In-gel digestion of the protein with trypsin or endoproteinase Glu-C (Roche Diagnostics, Indianapolis, IN) was carried out as described previously (29). The resultant peptides were separated using reversed phase HPLC on a microbore C8 column (Vydac, Hesperia, CA), and the collected fractions were subjected to scintillation counting. Individual peptides were subjected to covalent Edman degradation on a Sequelon AA membrane (PerSeptive Biosystems, Cambridge, MA) with a protein sequencer (model 492; Applied Biosystems, Foster City, CA). The anilinothiazolínoneamino acids were extracted from the filter with neat trifluoroacetic acid and scintillation counted. Radioactive profiles for each sequencing run were compared with theoretical peptide sequences derived from the protein.

Plasmids

Expression Vectors—To construct GalDBD-GRIP1, the GalDBD coding fragment from pGBT9·GRIPFL (32) was removed with *Hin*dIII-*Eco*RI and ligated to *Sma*I-*Eco*RI-cut pBS (Stratagene) giving rise to pBS·Gal4DBD. An *Eco*RI fragment of GRIP1 (amino acids 5–1462) from pGBT9 vector was then inserted into pBS·Gal4DBD. The entire Gal4 DBD-GRIP1 coding segment was then removed with *Xba*I-*Eco*RV and subcloned into the *Nhe*I-*Eco*RV site of commercially available pCMV vector (Stratagene). GalDBD-CBP has been described by Swope *et al.* (30). GST-GRIP1–479, GST-GRIP1–766, GST-GRIP184–766 and GST-GRIP766–1462 vectors for bacterial expression have been described by Webb *et al.* (7). The mutants GST-GRIPS736A and GST-GRIPS554A for mammalian expression were generated by polymerase chain reaction from parental vector GST-GRIP184–766 incorporating a mutagenic primer with *Pfu* polymerase (QuickChange site-directed mutagenesis kit, Stratagene). MPK-1 (CL100) expression vector was a gift of D. Stokoe (Cancer Center, University of California, San Francisco). MEKK97R expression vector has been described by Mansour *et al.* (31). pSG5-GRIPS736A was constructed from pSG5-GRIPFL (32) using mutagenic primer incorporation by polymerase chain reaction as described above. Gal-GRIPS736A and Gal-GRIPS554A are derivatives of Gal-GRIPFL vector and were mutated as indicated above. All the mutants were confirmed by sequencing, and generally, functional assays of two clones were carried out. pGFP-GRIP has green fluorescent protein fused to the amino terminus of GRIP expression vector and was a gift from Yihong Wan (University of Colorado, Health Sciences Center), and pGFP-GRIPS736A was constructed by removing a 1612-nucleotide *Bst*XI-*Bsp*EI fragment from pSG5-GRIPS736A and inserting it into the *Bst*XI-*Bsp*EI sites of pGFP-GRIP. The point mutation was confirmed by sequencing. The pSG5ER S118A mutant is a derivative of HEO (33) and is described by Webb *et al.* (7).

Reporters—GalRE-luc contains five Gal4 response elements upstream of a minimal adenovirus E1b promoter and has been previously described (34). It is devoid of the pUC AP1 site as described by Webb *et al.* (36). ERE-luc contains the 45-mer estrogen response element from the *Xenopus* vitellogenin A2 promoter driving expression of the luciferase gene cloned in a pUC vector devoid of the vector AP1 site (35) and has been previously described (36). The pΔTAT3-luc reporter has three glucocorticoid response elements from the regulatory region of tyrosine aminotransferase gene placed upstream of the minimal *Drosophila* distal alcohol dehydrogenase promoter (−33 to +55) driving luciferase expression (37).

Western Blot

20 million HeLa cells were transfected with 20 μg of GFP-GRIPwt or GFP-GRIPS736A expression vector, incubated overnight at 37 °C, exposed to EGF for 2 h, and harvested in lysis buffer (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 20 mM β-glycerophosphate, 0.1% Triton X-100), protease inhibitors, and Ser/Thr and Tyr phosphatase inhibitors (Sigma). The whole cell extract (50 μg) was resolved by 4–15% gradient polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Bio-Rad), and probed with anti-green fluorescent protein (GFP) antibodies (Convance, Berkeley, CA) or GRIP1 antibodies (Affinity BioReagents, Inc., Golden, CO) followed by the corresponding peroxidase-conjugated anti-IgG. Signals were visualized by the ECL detection kit (Amersham Pharmacia Biotech).

Fluorescence Microscopy

After transfections cells to be analyzed by microscopy were distributed on cover glass-containing wells, and a duplicate group of cells was treated for regular luciferase activity. Images were collected on a Zeiss Axioplan epifluorescence microscope using a 63×, 1.35 numerical aperture oil immersion objective and fluorescein isothiocyanate excitation and emission filters (Chroma Corp., Brattleboro, VT). Digital images were collected using a Xillix CCD camera and integration times from 50 to 150 ms. All the images presented in Fig. 6 were collected at the same integration times and processed identically.

RESULTS

A recent report suggests that growth factor stimulation of the transcriptional activation functions of CBP (38, 39) requires the domain of CBP that mediates p160 binding (10). We thus examined in transfected cells whether the p160 GRIP1 tethered to a reporter gene promoter by fusion to the heterologous Gal4 DNA binding domain (*Gal-GRIP1*, Fig. 1) could activate transcription in response to growth factors. EGF and TGF-α had no effect on reporter gene expression in the absence of GRIP1 (Fig. 1*A*), but these ligands of the EGF receptor each activated transcription 5–10-fold when GRIP1 was bound to the promoter. The GRIP1 response to EGF and TGF-α required the action of the ERK family of MAP kinases because it was abolished by overexpression of the MAP kinase phosphatase CL100, by a dominant negative MEK that specifically prevents activation of ERKs, and by PD98059, a specific inhibitor of MEK activation (Fig. 1, *B* and *C*). In control experiments EGF failed to stimulate reporter gene transcription mediated by a Gal4 fusion to the herpesvirus VP16 protein, and as expected, neither PD98059 nor CL100 nor dominant negative MEK was inhibitory (data not shown). Thus GRIP1 contains an EGF-regulated transcriptional activation function, and ERKs are a component of the pathway of activation.

To examine whether GRIP1 might serve as a direct substrate for ERKs, we prepared recombinant GRIP1 in *Escherichia coli* and incubated it with activated ERK2 *in vitro*. Among fragments that represent the entire protein, two fragments from amino acids 1–766 and 184–766 were strongly phosphorylated by MAP kinase (Fig. 2*A*). Fragment 1–479 was unreactive, and fragment 766–1462 was barely reactive. Thus GRIP1 serves as a MAP kinase substrate *in vitro*, and the major site(s) of phosphorylation lies within amino acids 184–766, most likely between 480 and 766.

The precise site of action of ERK MAP kinase was determined by using enzymatic protein digests and Edman analysis

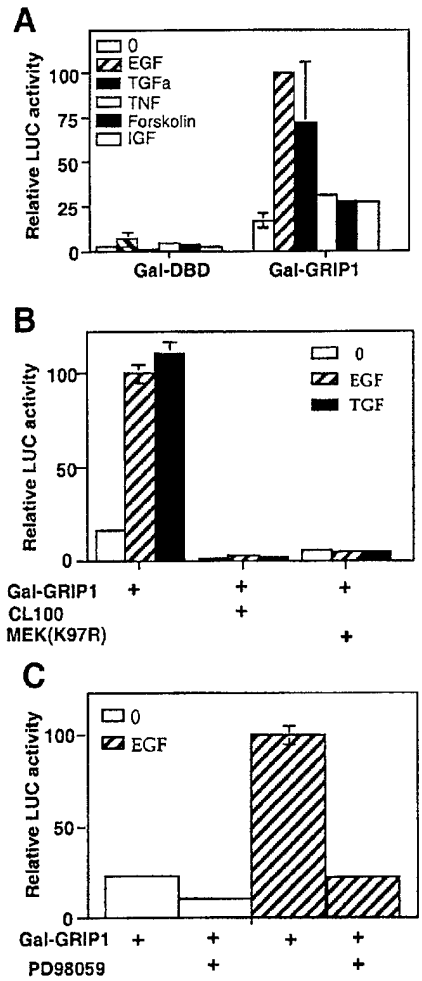

FIG. 1. **GRIP1 contains a growth factor-regulated and ERK kinase-mediated transcriptional activation function.** *A*, activity of a luciferase reporter gene regulated by Gal4 response elements (GalRE-luc) in cells expressing yeast Gal4 DNA binding domain by itself (*Gal-DBD*) or fused to GRIP1 (*Gal-GRIP1*) and treated with growth factors as indicated. *B* and *C*, EGF activation of GRIP1 is abolished by ERK inhibitors. Activity of GalRE-luc with expression vectors for Gal-GRIP1, dominant negative MEK (*MEK(K97R)*), the dual specificity phosphatase CL100 1B, or the MEK inhibitor PD98059. *TNF*, tumor necrosis factor; *IGF*, immunoglobulin growth factor.

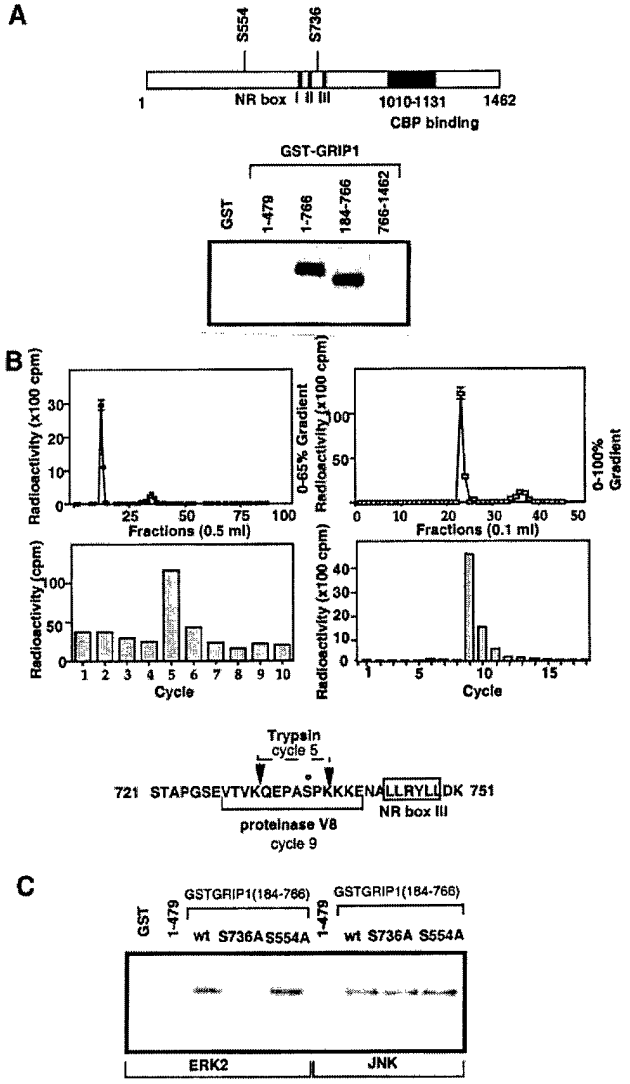

FIG. 2. **ERK phosphorylates GRIP1 on Ser-736 *in vitro*.** *A*, domain map of GRIP1 with the location of the nuclear receptor (NR) boxes, Ser-736 and Ser-554, and phosphorylation of *E. coli*-expressed fragments of GRIP1 by purified and activated ERK2. Products of the reaction with radiophosphate are shown after gel separation. A major phosphorylation site is located in the fragment spanning amino acids 184–766. *B*, mapping of the site of ERK phosphorylation on GRIP1. Edman analysis of the major peaks of radiophosphate on GRIP1 after incubation with ERK2 and digestion with either trypsin or endoproteinase Glu-C is shown. Both analyses predict Ser-736 as the major site of phosphorylation. *C*, confirmation that S736A is a major site of phosphorylation by ERK. Wild type (*wt*) GST-GRIP1-(184–766) and mutants S554A and S736A were incubated with ERK2 (*left panel*) or, as a control, with JNK1 (*right panel*), and the products were autoradiographed after gel separation.

of the phosphate-labeled GRIP1-(184–766). Each of these digests yielded a single major labeled peptide on HPLC, and the phosphate label was on the fifth amino acid of the tryptic peptide and the ninth of the V8 peptide (Fig. 2*B*). Each of these assays predicts that the major site of phosphorylation of GRIP1 is serine 736 (Fig. 2*B*), which is an ERK consensus site. To confirm the identity of the site, the phosphorylation reactions were repeated with wild type GRIP1-(184–766) and mutants in which Ser-736 or Ser-554, chosen because it resembles a MAP kinase site, was mutated to alanine. Phosphorylation was diminished on GRIP1 mutated on Ser-736 (Fig. 2*C*). The S554A mutant was phosphorylated as efficiently as wild type. In control experiments, JNK1, a MAP kinase with different specificity, phosphorylated all three substrates equally. We concluded that Ser-736 is a major target for phosphorylation of GRIP1 by ERK but not JNK *in vitro*.

To examine the role of Ser-736 *in vivo*, we repeated our studies of EGF activation of tethered GRIP1 using both wild type and mutants. EGF potentiated transcriptional activation mediated by wild type GRIP1 or the S554A mutant but was consistently weaker on the S736A mutant (Fig. 3*A*, *upper panel*). Despite this diminution of response, the Gal-GRIPS736A mutant was well expressed as detected by Western blots (not shown) and by its ability to serve as "bait" in a mammalian two-hybrid assay (Fig. 3*A*, *lower panel*). These results indicate that GRIP1 bound directly to the promoter requires Ser-736 for full activation by EGF.

We then tested the requirement for Ser-736 when GRIP1 functions as a coactivator for the PR activated by EGF signaling. We used a reporter gene with three response elements from the tyrosine aminotransferase promoter (TAT3-luc), which the PR activates 3-fold in the presence of EGF (Fig. 3*B*).

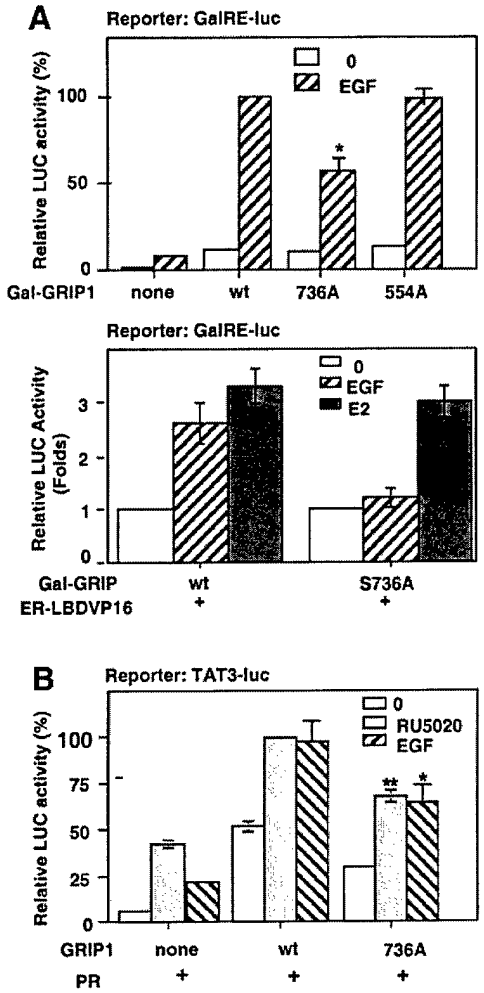

FIG. 3. **Mutation of Ser-736 to Ala decreases EGF-induced transcriptional activation by Gal-GRIP1 and diminishes EGF-induced coactivator functions.** *A, top panel*, expression of a GalRE-luc reporter gene activated by Gal-GRIP1 wild type or the indicated mutant and induced with EGF. *, $p < 0.028$ as compared with EGF-induced level of Gal-GRIPwt by standardized Student's *t* test. *Bottom panel*, comparison of the ability of Gal-GRIPwt and Gal-GRIPS736A to act as bait in a mammalian two-hybrid assay in which they are tethered on a GalRE-luc reporter gene and activated with an expression vector for the ligand binding domain of estrogen receptor fused to the VP16 activation domain (*ER-LBDVP16*) in the absence or presence of EGF or E2. *B*, expression of a PRE-luciferase reporter gene (*TAT3-luc*) activated by PR, EGF, or the progestin RU5020 as indicated and potentiated by wild type or S736A mutant of GRIP1. Values correspond to the mean ± %cv of triplicate determinations from a representative experiment that was performed separately at least three times. **, $p < 0.003$ as compared with the RU5020-induced coactivation by GRIPwt; *, $p < 0.01$ for EGF-induced coactivation by mutant GRIPS736A compared with EGF-induced levels by GRIPwt.

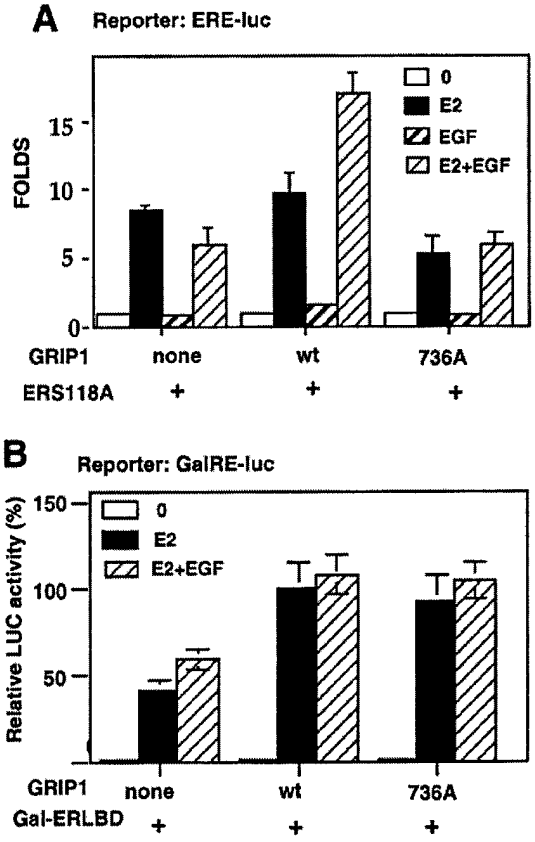

FIG. 4. *A*, effect of GRIP1 wild type and S736A on the expression of an ERE-luciferase reporter gene (*ERE-luc*) activated by ER-S118A and estrogen (*E2*) or EGF as indicated. *B*, effect of GRIP1 wild type and S736A on expression of a GalRE-luciferase reporter gene (*GalRE-luc*) activated by a Gal4-ER LBD fusion protein, estrogen, and EGF as indicated.

Wild type GRIP1 as well as the S554A mutant potentiated PR-mediated transcription of the reporter gene approximately 4-fold after activation with EGF. Both the hormone-liganded and constitutive activity of the PR were potentiated, as is frequently observed for nuclear receptors with overexpressed coactivators (data not shown). The S736A mutant was only half as effective as wild type in potentiating PR action. In control experiments with hormone-activated glucocorticoid receptor, which does not respond to EGF, both wild type and S736A GRIP1 potentiated receptor action to similar extents (data not shown), indicating that the Ser-736 mutant of GRIP1 retains full function in some contexts. S736A GRIP1 was, however, less able than wild type GRIP1 to potentiate progestin-activated PR. Some of this deficit may reflect a role for low level activation of ERK or some other kinase on the inducible functions of GRIP1 on progestin-PR, even in the absence of deliberate EGF stimulation (data not shown). For other potential explanations see Lange *et al.* (40). Thus S736A GRIP1 retains full ability to serve as a coactivator in some circumstances but is deficient in EGF-regulated coactivator function on the PR.

Estrogen receptor activation of transcription from a reporter gene regulated by a consensus ERE is potentiated by EGF to varying extents depending on cells and culture conditions. We tested the ability of an ER S118A, mutated in the major site of EGF-dependent phosphorylation, for response to EGF with GRIP1. Without elevated GRIP1, EGF had little or no effect on transcriptional activation by ER S118A either in the absence or presence of estrogen (Fig. 4*A*, *E2*). With elevated GRIP1, EGF potentiated both unliganded and estrogen-liganded ER S118A action 2-fold. Interestingly, the S736A GRIP1 mutant was unable to support an EGF response. The isolated ER AF-2 function, which can be tested as a fusion of the ER LBD to the yeast Gal4 DNA binding domain tethered on a Gal4 response element, does not respond to EGF (Fig. 4*B*). The S736A mutant was undiminished in its ability to function as a coactivator for isolated ER AF-2. Thus, S736A retains full coactivator function with the isolated ER LBD but is deficient in coactivator function for full-length ER S118A.

As noted above, GRIP1 is also bound to and synergizes with CBP/p300 (10, 11, 41). It is possible that EGF regulates GRIP1 coactivator activities by affecting the efficiency of this interaction. Indeed, it was recently reported that activated MEK in-

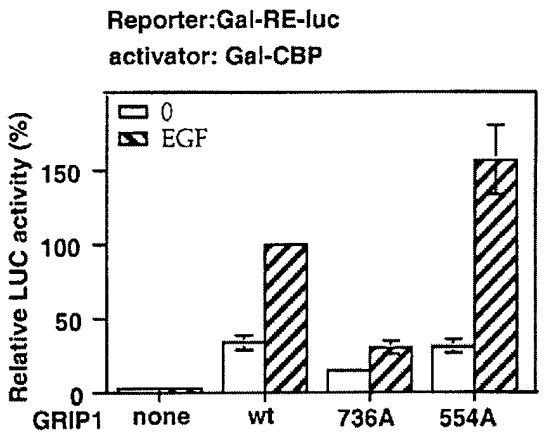

FIG. 5. **Mutation of Ser-736 to Ala impairs the ability of GRIP1 to serve as an EGF-inducible coactivator for Gal-CBP.** Expression of the GalRE-luc reporter gene when activated by a fusion of the p160 binding domain of CBP to yeast Gal4 DNA binding domain (*Gal-CBP*), wild type or mutant versions of GRIP1, and EGF is as indicated.

creased the association of CBP and the p160 AIB1 (42). Although expression of activated MEK led to phosphorylation of AIB1 the site(s) was not analyzed. We therefore examined the ability of wild type and S736A GRIP1 to potentiate transcription mediated by CBP. We tethered CBP directly to a promoter by fusion to a heterologous Gal4 DNA binding domain. In the absence of overexpressed GRIP1 the CBP domain weakly activates transcription (Fig. 5). Coexpression of wild type GRIP1 or the S554A mutant markedly potentiates transcription, which becomes sensitive to further induction by EGF. The S736A mutant of GRIP1 is, however, compromised in its ability to activate tethered CBP, and the EGF response is decreased.

To confirm that the defects of the S736A mutant are not due to loss of stability or nuclear localization ability, we fused both wild type and mutant GRIP1 to GFP and monitored GRIP1 expression levels and nuclear localization. Both wild type and Ser-736 were well expressed and both localized to the nucleus (Fig. 6, *top panel*). Western blots indicated equal expression of wild type and mutant (Fig. 6, *middle panel*). When fused to GFP wild type GRIP1 had full coactivator action with tethered CBP and EGF, but the S736A mutant was diminished (Fig. 6, *bottom panel*). Thus the S736A mutation does not affect GRIP1 expression or localization yet has a specific effect on EGF-responsive coactivator function.

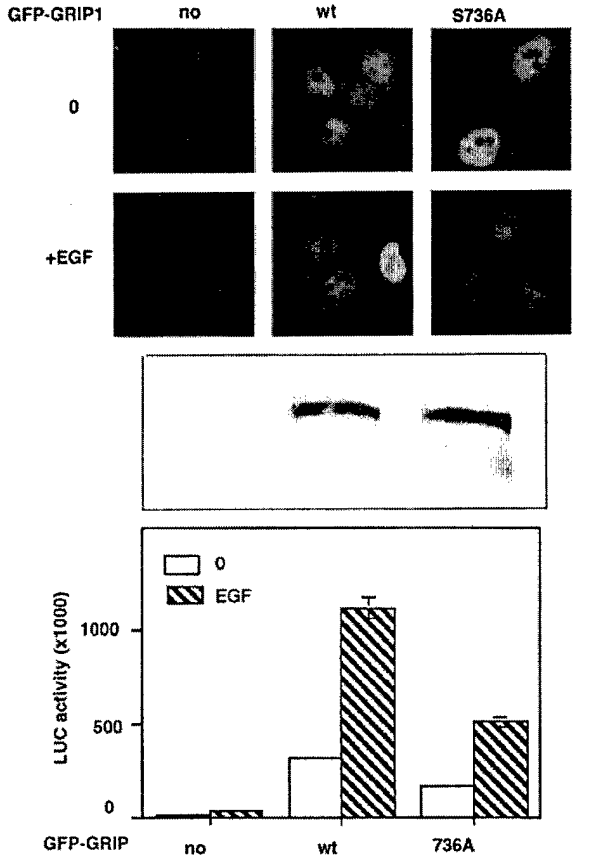

FIG. 6. **Expression and localization of GRIP1 is unaffected by the S736A mutation.** *Top panels*, fluorescence from fusions of GFP to GRIP1 wild type or S736A expressed in HeLa cells either untreated or EGF-treated as indicated. *Middle panel*, Western blot of the transfected cell extracts probed with anti-GFP. *Lower panel*, effect of GFP-GRIP1 wild type or S736A on expression of a GalRE-luc reporter gene activated with Gal-CBP and EGF as indicated.

DISCUSSION

The observations above indicate that EGF-activated ERK MAP kinase potentiates the transcriptional activation functions of GRIP1. Furthermore, activated ERKs phosphorylate GRIP1 on serine 736 *in vitro*. Mutation of Ser-736 to alanine substantially reduces the ability of GRIP1 to enhance transcription of the EGF-activated PR and of the estrogen- and EGF-potentiated ER. Because the mutant GRIP1 is fully able to function as a coactivator for the GR and for the isolated ER LBD AF-2, receptors that do not respond to EGF, it appears that the defect of S736A is limited and may indeed be specific for GRIP1 coactivator function with EGF-responsive steroid receptors or other transcription factors. In sum these observations suggest that EGF-activated ERKs increase selected coactivator functions of GRIP1 through a pathway requiring the integrity and most likely the phosphorylation of Ser-736.

While this study was in preparation, it was reported that AIB1, another member of the p160 family, contained a transcriptional function that was activated by transfection with constitutive MEK, a potent activator of ERKs (42). Activation by MEK was independent of the D2 domain of AIB1, suggesting that AD1 was responsible. Indeed fragments of AIB1 that contain AD1 and the nuclear receptor boxes are phosphorylated in MEK-transfected cells, suggesting that the target of MEK activation is in this region and may require AD1 for an output. Interestingly, a deletion of AD1 of GRIP1 compromises the EGF response with ER and PR (data not shown).

Activation by MEK was also reported to increase the binding of CBP with AIB1 fragments that contain the AD1 domain. Our studies complement this observation in that we observed an EGF-induced increase in the functional interaction between the p160 binding domain of CBP tethered to DNA by fusion to the Gal4 DNA binding domain and GRIP1. The interaction is compromised by the Ser-736 mutation, suggesting a direct role for this site in the interaction. Sequence comparison of AIB1 and GRIP1 indicates that the Ser-736 residue is not conserved. Therefore mechanistic comparison must await the identification of the MEK-phosphorylated residues in AIB1 and their mutational analysis.

The EGF pathway to GRIP1 and thence to transcription factors that recruit GRIP1 may provide a biological means to coordinate EGF effects at many different transcription factors and their target genes. It has been well established that EGF initiates a signal transduction phosphorylation cascade that leads to transcription factors such as Elk-1, ER, and PR. Direct phosphorylation of the transcription factors is required for wild type Elk-1 and ER response to EGF. Nonetheless, there are

hints that in the absence of direct phosphorylation, such as when the ER Ser-118 is mutated to glutamate, an EGF response persists. Thus, we suggest that for an EGF-induced signal cascade to be fully effective the cascade may need to target both the transcription factor and the recruited coactivator. This double requirement may prevent random biological noise from activation of the EGF response.

Acknowledgments—We thank Paul Webb, Branka Kovacic, and David Stokoe for plasmids and helpful discussion.

REFERENCES

1. Xu, L., Glass, C. K., and Rosenfeld, M. G. (1999) *Curr. Opin. Genet. Dev.* **9,** 140–147
2. Feng, W., Ribeiro, R. C., Wagner, R. L., Nguyen, H., Apriletti, J. W., Fletterick, R. J., Baxter, J. D., Kushner, P. J., and West, B. L. (1998) *Science* **280,** 1747–1749
3. Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) *Genes Dev.* **12,** 3343–3356
4. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) *Cell* **95,** 927–937
5. Hong, H., Darimont, B. D., Ma, H., Yang, L., Yamamoto, K. R., and Stallcup, M. R. (1999) *J. Biol. Chem.* **274,** 3496–3502
6. Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., and Milburn, M. V. (1998) *Nature* **395,** 137–143
7. Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M. P., Chen, D., Huang, S. M., Subramanian, S., McKinerney, E., Katzenellenbogen, B. S., Stallcup, M. R., and Kushner, P. J. (1998) *Mol. Endocrinol.* **12,** 1605–1618
8. Ma, H., Hong, H., Huang, S. M., Irvine, R. A., Webb, P., Kushner, P. J., Coetzee, G. A., and Stallcup, M. R. (1999) *Mol. Cell. Biol.* **19,** 6164–6173
9. Voegel, J. J., Heine, M. J., Tini, M., Vivat, V., Chambon, P., and Gronemeyer, H. (1998) *EMBO J.* **17,** 507–519
10. Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) *Cell* **85,** 403–414
11. Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* **387,** 677–684
12. Bannister, A. J., and Kouzarides, T. (1996) *Nature* **384,** 641–643
13. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) *Cell* **87,** 953–959
14. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) *Cell* **90,** 569–580
15. Li, H., Gomes, P. J., and Chen, J. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 8479–8484
16. Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) *Nature* **389,** 194–198
17. Imhof, A., Yang, X. J., Ogryzko, V. V., Nakatani, Y., Wolffe, A. P., and Ge, H. (1997) *Curr. Biol.* **7,** 689–692
18. Chen, H., Lin, R. J., Xie, W., Wilpitz, D., and Evans, R. M. (1999) *Cell* **98,** 675–686
19. Chen, D., Ma, H., Hong, H., Koh, S. S., Huang, S. M., Schurter, B. T., Aswad, D. W., and Stallcup, M. R. (1999) *Science* **284,** 2174–2177
20. Weigel, N. L., and Zhang, Y. (1998) *J. Mol. Med.* **76,** 469–479
21. Bai, W., Rowan, B. G., Allgood, V. E., O'Malley, B. W., and Weigel, N. L. (1997) *J. Biol. Chem.* **272,** 10457–10463
22. Kato, S., Kitamoto, T., Masuhiro, Y., and Yanagisawa, J. (1998) *Oncology* **55,** Suppl. 1, 5–10
23. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995) *Science* **270,** 1491–1494
24. Ignar-Trowbridge, D. M., Pimentel, M., Teng, C. T., Korach, K. S., and McLachlan, J. A. (1995) *Environ. Health Perspect.* **7,** 35–38
25. Bunone, G., Briand, P. A., Miksicek, R. J., and Picard, D. (1996) *EMBO J.* **15,** 2174–2183
26. Ignar, T. D., Pimentel, M., Parker, M. G., McLachlan, J. A., and Korach, K. S. (1996) *Endocrinology* **137,** 1735–1744
27. Endoh, H., Maruyama, K., Masuhiro, Y., Kobayashi, Y., Goto, M., Tai, H., Yanagisawa, J., Metzger, D., Hashimoto, S., and Kato, S. (1999) *Mol. Cell. Biol.* **19,** 5363–5372
28. Webb, P., Lopez, G. N., Greene, G. L., Baxter, J. D., and Kushner, P. J. (1992) *Mol. Endocrinol.* **6,** 157–167
29. Hellman, U., Wernstedt, C., Gonez, J., and Heldin, C. H. (1995) *Anal. Biochem.* **224,** 451–455
30. Swope, D. L., Mueller, C. L., and Chrivia, J. C. (1996) *J. Biol. Chem.* **271,** 28138–28145
31. Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., and Ahn, N. G. (1994) *Science* **265,** 966–970
32. Ding, X. F., Anderson, C. M., Ma, H., Hong, H., Uht, R. M., Kushner, P. J., and Stallcup, M. R. (1998) *Mol. Endocrinol.* **12,** 302–313
33. Tora, L., Mullick, A., Metzger, D., Ponglikitmongkol, M., Park, I., and Chambon, P. (1989) *EMBO J.* **8,** 1981–1986
34. Saatcioglu, F., Lopez, G., West, B. L., Zandi, E., Feng, W., Lu, H., Esmaili, A., Apriletti, J. W., Kushner, P. J., Baxter, J. D., and Karin, M. (1997) *Mol. Cell. Biol.* **17,** 4687–4695
35. Lopez, G., Schaufele, F., Webb, P., Holloway, J. M., Baxter, J. D., and Kushner, P. J. (1993) *Mol. Cell. Biol.* **13,** 3042–3049
36. Webb, P., Lopez, G. N., Uht, R. M., and Kushner, P. J. (1995) *Mol. Endocrinol.* **9,** 443–456
37. Iniguez-Lluhi, J. A., and Pearce, D. (2000) *Mol. Cell. Biol.* **20,** 6040–6050
38. Janknecht, R., and Nordheim, A. (1996) *Biochem. Biophys. Res. Commun.* **228,** 831–837
39. Liu, Y. Z., Chrivia, J. C., and Latchman, D. S. (1998) *J. Biol. Chem.* **273,** 32400–32407
40. Lange, C. A., Richer, J. K., Shen, T., and Horwitz, K. B. (1998) *J. Biol. Chem.* **273,** 31308–31316
41. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) *Recent Prog. Horm. Res.* **52,** 141–165
42. Font de Mora, J., and Brown, M. (2000) *Mol. Cell. Biol.* **20,** 5041–5047